Opposition à un brevet européen

A l'Office européen des brevets

Arrêts de tabulation

réservé à l'OEB

I. Brevet attaqué

N° de l'oppos. OPPO (1)

Numéro du brevet	656 786
Numéro de la demande	93909679.8
Date de la mention de la délivrance (art. 97(4), 99(1) CBE)	15.09.04

Titre de l'invention

Utilisation d'extraits des phyto-oestrogènes isoflavones de soja ou de trèfle

II. Unique ou premier titulaire du brevet cité dans le fascicule du brevet

NOVOGEN RESEARCH Pty Ltd

Référence de l'opposant ou du mandataire (max. 15 caractères ou espaces) OREF

III. Opposant

OPPO (2)

Nom

Adresse

Laboratoires ARKOPHARMA
1ère Avenue 2709
MLID de CARROS LE BROC
06510 CARROS

Zur Kasse
€61,- 1-

Etat du domicile ou du siège FRANCE

Téléphone/Télex/Téléfax

Opposition conjointe [X] Autres opposants, voir feuille additionnelle

IV. Représentation

1. **Mandataire** (N'indiquer qu'un seul mandataire à qui toute correspondance doit être adressée) OPPO (9)

Nom Jacques WARCOIN

Adresse professionnelle

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Autre(s) mandataire(s) [ ] (voir feuille additionnelle/pouvoir) OPPO (5)

2. **Employé(s)** de l'opposant muni(s) d'un pouvoir conformément à l'art. 133(3) CBE pour la présente procédure d'opposition — Nom(s):

Pouvoir(s) [X] considéré comme non nécessaire

Pour 1./2. [ ] enregistré(s) sous le n°

[ ] ci-joint(s)

réservé à l'OEB

V. L'opposition est formée contre le brevet

— dans son ensemble [X]

— dans la limite des revendications n°s

VI. Motifs d'opposition :

L'opposition est fondée sur les motifs mentionnés ci-après :

(a) l'objet du brevet européen n'est pas brevetable (art. 100(a) CBE), pour les motifs suivants :

— défaut de nouveauté (art. 52(1) et 54 CBE) [X]

— défaut d'activité inventive (art. 52(1) et 56 CBE) [X]

— autres motifs excluant la brevetabilité, à savoir art. [ ]

(b) le brevet européen n'expose pas l'invention de façon suffisamment claire et complète pour qu'un homme du métier puisse l'exécuter (art. 100(b) CBE ; cf. art. 83 CBE). [X]

(c) l'objet du brevet européen s'étend au-delà du contenu de la demande/demande initiale telle qu'elle a été déposée (art. 100(c) CBE; cf. art. 123(2) CBE). [ ]

VII. Exposé des faits et motifs

(règle 55(c) CBE)
fait l'objet de la déclaration ci-jointe (Annexe 1) [X]

VIII. Autres requêtes

A titre de précaution, l'opposant demande l'établissement d'une procédure orale selon l'article 116 CBE.

IX. Justifications invoquées

réservé à l'OEB

ci-jointes = ☒

sera (seront) produit(s) ultérieurement = ☐

VOIR MEMOIRE D'OPPOSITION

A. **Publications :** — Date de la publication

1

en particulier, page/colonne/ligne/fig. :

2

en particulier, page/colonne/ligne/fig. :

3

en particulier, page/colonne/ligne/fig. :

4

en particulier, page/colonne/ligne/fig. :

5

en particulier, page/colonne/ligne/fig. :

6

en particulier, page/colonne/ligne/fig. :

7

en particulier, page/colonne/ligne/fig. :

suite sur feuille additionnelle ☐

B. **Autres justifications**

Autres indications sur feuille additionnelle ☐

réservé à l'OEB

X. Paiement de la taxe d'opposition

[X] comme indiqué sur le bordereau de règlement de taxes et de frais (OEB Form 1010) ci-joint

[ ]

XI. Relevé des pièces

Annexe n°		Nombre d'exemplaires	
0	[X] Formulaire d'opposition	2 (2 au moins)	
1	[X] Exposé des faits et motifs (cf. VII.)	2 (2 au moins)	
2	Copies des justifications invoquées (cf. IX.)		
2a	[X] — Publications	2 (2 au moins pour chaque)	
2b	[ ] — Autres pièces	(2 au moins pour chaque)	
3	[ ] Pouvoir(s) signé(s) (cf. IV.)		
4	[X] Bordereau de règlement de taxes et de frais (cf. X.)	4	
5	[ ] Chèque		
6	[X] Feuille(s) additionnelle(s)	4 (2 au moins pour chaque)	
7	[ ] Autres pièces (veuillez préciser)		

XII. Signature de l'opposant ou du mandataire

Lieu Paris,

Date le 15 juin 2005

Prière de dactylographier le nom du (des) signataire(s). S'il s'agit d'une personne morale, la position occupée au sein de celle-ci par le ou les signataire(s) sera indiquée à la machine à écrire.

Feuilles additionnelles

Numéro du brevet	EP 656786
Numéro de la demande	93909679.8
Date de la mention de la délivrance (art.97(4), 99(1) CBE)	15.09.2004
Titre de l'invention	Utilisation d'extraits des phyto-oestrogènes isoflavones de soja ou de trèfle

Autres opposants :

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Nom	LABORATOIRE de Phytothérapie et d'herboristerie L.P.H.
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Nom	TOURNAY BIOTECHNOLOGIES
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Nom	LEA INSTITUT VITAL
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Nom	LABORATOIRE Théramex
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Nom	JUVA
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Nom	NUTRINOV
Adresse	Rue Siméon Poisson - Campus de Ker Lann - 35170 Bruz - France

Nom	NUTRITION ET SANTE
Adresse	Route de Castelnaudary - 31250 Revel - France

Mémoire d'opposition contre le brevet EP 656 786

Demande de brevet n° 93909679.8

Titulaire : NOVOGEN RESEARCH Pty Ltd

Titre : UTILISATION D'EXTRAITS DES PHYTO-OESTROGENES ISOFLAVONES DE SOJA OU DE TREFLE

Date de publication de mention de la délivrance : 15 septembre 2004

Opposants : LABORATOIRES ARKOPHARMA
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L'opposition est formée à l'encontre de toutes les revendications 1 à 11 du brevet EP 656 786 et est basée sur l'article 100 (a) CBE pour les raisons que l'invention revendiquée ne présente pas de nouveauté au sens de l'article 54 CBE, ni n'implique d'activité inventive au sens de l'article 56 CBE.

L'opposition se fonde par ailleurs sur l'article 100 (b) CBE.

L'opposant requiert la révocation totale du brevet EP 656 786. A titre de précaution, l'opposant demande l'établissement d'une procédure orale selon l'article 116 CBE.

FAITS ET JUSTIFICATIONS A L'APPUI DES MOTIFS D'OPPOSITION :

Etat de la Technique à l'appui de l'opposition :

Les documents ci-après sont cités comme état de la technique au titre de l'article 54 (2) CBE contre l'ensemble des revendications.

D1 : "Oestrogenic effects of plant foods in postmenopausal women" Wilcox G. et al., *British Med. J.*, 1990, 301, pages 905-906;
D2 : "Herbal help to avoid menopause symptoms" Beckham N., *Australian Wellbeing*, 1988, No. 29, pages 74-76 ;
D3 : "Dietary phyto-oestrogens and the menopause in Japan" Adlercreutz H. et al., *The Lancet*, 1992, Vol. 339, page 1233 ;
D4 : "Western diet and western diseases: some hormonal and biochemical mechanisms and associations" Adlercreutz H. et al., *Scand. J. Clin. Lab. Invest.*, 1990, 50, Suppl 201, pages 3-23;
D5 : "Dietary phytoestrogens and cancer: in vitro and in vivo studies" Adlercreutz H. et al., *J. Steroid Biochem. Molec. Biol.*, 1992, Vol. 41, No. 3-8, pages 331-337;
D6 : "Urinary excretion of lignans and isoflavonoid phytoestrogens in Japanese men and women consuming a traditional Japanese diet" Adlercreutz H. et al., *Am. J. Clin. Nutr.*, 1991, Vol. 54, pages 1093-1100 ;
D7 : "Role of plant estrogens in normal and estrogen-related altered growth of the mouse prostate" Mäkelä S. et al., *Proc. Euro. Food Tox.*, 1991, Zürich ,Switzerland, III, pages 135-139;
D8 : EP 426 998, demande de brevet publiée le 15 mai 1991 ;
D9 : EP 135 172, demande de brevet publiée le 27 mars 1985 ;
D10 : "Determination of isoflavones in soybean flours, protein concentrates, and isolates" Eldridge A. C., *J. Agric. Food Chem.*, 1982, Vol. 30, pages 353-355 ;
D11 : Résumé de JP 62106016, demande de brevet publiée le 16 mai 1987 ;
D12 : "Naturally occuring oestrogens in foods – A review" Price K. R.. et al., *Food additive and contaminants*, 1985, Vol. 2, No.2, pages 73-106.

D1 à D12 sont des documents de l'art antérieur au titre de l'article 54 (2) CBE envers l'ensemble des revendications 1 à 11 du brevet EP 656 786.

Des copies des documents D1 à D12 sont annexées en double exemplaire.

Objet du brevet opposé :

- La revendication 1 indépendante concerne l'utilisation d'un extrait du phyto-œstrogène isoflavone de soja ou de trèfle pour fabriquer un médicament pour administration sous forme de dosage unitaire pour le traitement du syndrome prémenstruel, des symptômes associés à la ménopause ou du cancer de la prostate.

- Les revendications 2 à 11 sont des revendications dépendantes de la revendication 1, donnant des précisions sur le fait d'ajouter un excipient (revendication 2), la source du phyto-œstrogène (revendications 3 à 5), la nature, le ratio et la dose des isoflavones (revendications

6, 7 et 8), la durée d'administration du traitement (revendication 9), les constituants de l'extrait (revendication 10), et la forme de dosage unitaire (revendication 11).

Arguments :

I. Article 100 (a)

Revendication 1

Absence de nouveauté au sens de l'article 54(2) CBE sur la base de D1

1. De D1 est connue l'augmentation de la maturation vaginale par l'administration de phyto-œstrogènes de soja, tels que les isoflavones (page 905, colonne de gauche), chez des femmes post-ménopausées. D1 est une étude sur les effets de trois types d'aliments (soja, trèfle, lin), sur les femmes post-ménopausées, qui divulgue que la maturation vaginale est un indicateur du caractère oestrogénique chez l'être humain (page 906, dernier paragraphe : « Comment »). D1 conclut page 906, colonne de droite, que les phyto-œstrogènes sont en mesure de soulager les symptômes de la ménopause.

2. Même si D1 ne mentionne pas expressément que les phyto-œstrogènes isoflavones de soja sont incorporés dans un médicament pour administration sous forme de dosage unitaire, une telle caractéristique est implicitement contenue dans D1 de par la nature même de l'application visée, à savoir traiter les symptômes associés à la ménopause.

3. Par conséquent, toutes les caractéristiques de la revendication 1 sont connues de D1, dénuant l'objet de la revendication 1 de nouveauté.

4. L'enseignement de D1 divulguant l'ensemble des caractéristiques techniques de la revendication 1 est corroboré par le titulaire du brevet lui-même qui admet en tant qu'art antérieur une étude décrivant les effets bénéfiques des phyto-œstrogènes pour traiter les maladies occidentales (« Western diseases »), en particulier le traitement de symptômes de la ménopause par administration de phyto-œstrogènes provenant notamment du soja. Cette étude est sans aucun doute le document D1 (Cf. texte du brevet tel que délivré page 4 lignes 47 à 51).

Absence de nouveauté au sens de l'article 54(2) CBE sur la base de D2

5. De D2 est connu le traitement de divers problèmes liés à la ménopause par l'administration de phyto-œstrogènes de soja ou de trèfle, tels que la génistéine (Cf. page 75, colonne de droite, dernier paragraphe, qui mentionne spécifiquement la génistéine). La génistéine est une isoflavone connue que l'on trouve notamment dans le soja ou le trèfle.

6. Même si D2 ne mentionne pas expressément que les phyto-œstrogènes isoflavones de soja ou de trèfle peuvent être incorporés dans un médicament pour administration sous forme de

dosage unitaire, une telle caractéristique est implicitement contenue dans D2 de par la nature même de l'application visée, à savoir traiter les symptômes associés à la ménopause.

7. Par conséquent, toutes les caractéristiques de la revendication 1 sont connues de D2, dénuant l'objet de la revendication 1 de nouveauté.

Absence de nouveauté au sens de l'article 54(2) CBE sur la base de D3

8. D3 divulgue que les femmes japonaises ont moins de symptômes associés à la ménopause, tels que les bouffées de chaleur, que les femmes occidentales. D3 divulgue également que les japonais, qui ont un régime alimentaire traditionnel faible en graisses, ont un taux d'excrétion très élevé d'isoflavones phyto-œstrogènes dans leurs urines. D3 mentionne notamment l'excrétion importante de génistéine, de daidzéine et d'équol. D3 précise que l'excrétion dans les urines des japonais d'un taux élevé d'isoflavones est directement liée à l'ingestion de produits à base de soja. D3 conclut que ces taux élevés d'isoflavones phyto-œstrogènes peuvent avoir des effets bénéfiques chez les Japonais, et notamment chez les femmes post-ménopausées qui ont en général des taux faibles en œstrogènes. D3 conclut encore que ces taux élevés d'isoflavones phyto-œstrogènes expliquent en partie le fait que les femmes japonaises souffrent très peu des bouffées de chaleur, et plus généralement des symptômes de la ménopause.

9. Même si D3 ne mentionne pas expressément que les isoflavones phyto-œstrogènes de soja peuvent être incorporés dans un médicament pour administration sous forme de dosage unitaire, une telle caractéristique est implicitement contenue dans D3 de par la nature même de l'application visée, à savoir traiter les symptômes associés à la ménopause

10. Par conséquent, toutes les caractéristiques de la revendication 1 sont connues de D3, dénuant l'objet de la revendication 1 de nouveauté.

Absence de nouveauté au sens de l'article 54(2) CBE sur la base de D4

11. De D4 est connu l'effet protecteur des isoflavones phyto-œstrogènes sur le cancer de la prostate. D4 divulgue en effet que ces isoflavones permettent d'inhiber la croissance ou de limiter les risques de développement du cancer de la prostate (Cf. notamment page 14 colonne de gauche, et 1er paragraphe colonne de droite). D4 divulgue également que l'excrétion dans les urines des japonais d'un taux élevé d'isoflavones, telles que la daidzéine et l'équol, est directement liée à l'ingestion de produits à base de soja. D4 conclut que les isoflavones phyto-œstrogènes permettent le ralentissement du développement des cellules cancéreuses, et que l'administration de produits à base de soja peut ainsi avoir un effet bénéfique sur le traitement du cancer de la prostate.

12. Même si D4 ne mentionne pas expressément que les phyto-œstrogènes isoflavones de soja peuvent être incorporés dans un médicament pour administration sous forme de dosage unitaire, une telle caractéristique est implicitement contenue dans D4 de par la nature même de l'application visée, à savoir traiter le cancer de la prostate.

13. Par conséquent, toutes les caractéristiques de la revendication 1 sont connues de D4, dénuant l'objet de la revendication 1 de nouveauté.

Absence de nouveauté au sens de l'article 54(2) CBE sur la base de D5

14. De D5 est connu l'effet protecteur des isoflavones phyto-œstrogènes au regard du cancer de la prostate (Cf. notamment page 331, colonne de droite, avant-dernier et dernier paragraphes). D5 divulgue également que l'excrétion dans les urines chez l'être humain d'un taux élevé d'isoflavones, tels que la génistéine et la daidzéine, et de lignanes est directement liée à l'ingestion de produits alimentaires, en particulier de produits à base de soja.

15. Même si D5 ne mentionne pas expressément que les phyto-œstrogènes isoflavones de soja peuvent être incorporés dans un médicament pour administration sous forme de dosage unitaire, une telle caractéristique est implicitement contenue dans D5 de par la nature même de l'application visée, à savoir traiter le cancer de la prostate.

16. Par conséquent, toutes les caractéristiques de la revendication 1 sont connues de D5, dénuant l'objet de la revendication 1 de nouveauté.

Absence de nouveauté au sens de l'article 54(2) CBE sur la base de D6

17. De D6 est connu le traitement et la prévention de cancers hormono-dépendants, tels que le cancer de la prostate, par l'administration d'isoflavones phyto-œstrogènes provenant notamment de produits à base de soja (Cf. notamment page 1093, colonne de droite, 2ème et 3ème paragraphes ; page 1097, colonne de droite, 3ème paragraphe et page 1098). D6 mentionne que la génistéine, la daidzéine et l'équol sont des isoflavones phyto-œstrogènes provenant du soja.

18. Même si D6 ne mentionne pas expressément que les phyto-œstrogènes isoflavones de soja peuvent être incorporés dans un médicament pour administration sous forme de dosage unitaire, une telle caractéristique est implicitement contenue dans D6 de par la nature même de l'application visée, à savoir traiter le cancer de la prostate.

19. Par conséquent, toutes les caractéristiques de la revendication 1 sont connues de D6, dénuant l'objet de la revendication 1 de nouveauté.

Absence de nouveauté au sens de l'article 54(2) CBE sur la base de D7

20. D7 divulgue que des souris qui sont alimentées avec des produits à base de soja excrètent dans leur urine des quantités importantes de phyto-œstrogènes, tels que les isoflavonoïdes (Cf. page 136 le tableau 1, et le dernier paragraphe « Results », ainsi que page 139, premières lignes). D7 divulgue en outre que ces phyto-œstrogènes de soja permettent d'inhiber le développement des néoplasies prostatiques (Cf. en particulier page 138 5° « Effect of dietary soy on the development of dysplastic changes in the prostate of neoDES mice » où est précisé que l'ingestion de soja permet d'inhiber le développement de changements dysplasiques qui sont considérés comme étant de nature prémaligne Cf. page 136, 5ème paragraphe « Neonatal estrogenization of the mouse »). D7 conclut, page 137, que les phyto-œstrogènes de soja, dont les isoflavones qui sont expressément cités, permettent d'inhiber le développement du cancer de la prostate.

21. Même si D7 ne mentionne pas expressément que les phyto-œstrogènes isoflavones de soja peuvent être incorporés dans un médicament pour administration sous forme de dosage unitaire, une telle caractéristique est implicitement contenue dans D7 de par la nature même de l'application visée, à savoir traiter le cancer de la prostate.

22. Par conséquent, toutes les caractéristiques de la revendication 1 sont connues de D7, dénuant l'objet de la revendication 1 de nouveauté.

Absence de nouveauté au sens de l'article 54(2) CBE sur la base de D8

23. De D8 sont connus des extraits d'isoflavones de soja, le malonate de génistine et le malonate de daidzine, qui peuvent être incorporés dans des produits alimentaires ou cosmétiques (Cf. notamment page 3, lignes 46 à 48 et revendication 4). La génistine et la daidzine sont des phyto-œstrogènes connus du soja, et sont ainsi utilisés à titre de compléments alimentaires dans D8.

24. Le texte du brevet opposé EP 656 786 tel que délivré précise page 7, lignes 23 à 24, que les médicaments peuvent se présenter notamment sous forme de compléments alimentaires, de préparations pharmaceutiques, d'additifs alimentaires ou d'aliments contenant les phyto-œstrogènes actifs selon la présente invention. Le texte précise en outre page 7, ligne 28, que les médicaments sont de préférence présentés sous forme de remèdes ou traitements à base d'herbes.

25. Au vu de ce passage du brevet opposé, la revendication 1 du brevet opposé peut être interprétée à la lumière de la description comme l'utilisation d'un extrait du phyto-œstrogène isoflavone de soja ou de trèfle pour fabriquer un additif ou complément alimentaire. Par conséquent, la revendication 1 n'est pas nouvelle au regard de D8.

Absence d'activité inventive au sens de l'article 56 CBE sur la base de l'un des documents choisis parmi D1, D2, D3, D4, D5, D6, D7, ou D8, ou leurs combinaisons

26. Si, par impossible, la revendication 1 venait à être considérée comme nouvelle au vu de l'un des documents choisis parmi D1, D2, D3, D4, D5, D6, D7, ou D8, il y a alors lieu de considérer la revendication 1 comme n'impliquant pas d'activité inventive.

Absence d'activité inventive au sens de l'article 56 CBE sur la base de D9 en combinaison avec D10

27. De D9 est connu le traitement de l'ostéoporose par l'administration d'un médicament sous forme de dosage unitaire (une composition pharmaceutique) contenant la daidzéine (R=OH dans la formule). D9 divulgue également que l'ostéoporose est un trouble ou une maladie très fréquente chez les femmes post-ménopausées, signifiant ainsi que l'ostéoporose est un symptôme ou problème associé à la ménopause. D9 divulgue en outre page 2, lignes 10 à 15, que la daidzéine a une activité oestrogénique.

28. Ainsi, l'utilisation selon la revendication 1 se distingue de D9 en ce que l'isoflavone est extraite de plantes de soja ou de trèfle.

29. D10 enseigne que la daidzéine est l'une des principales isoflavones qui peuvent être extraites du soja. Un homme du métier savait donc à la date dont bénéficie l'objet de la revendication 1 que la daidzéine n'a pas besoin d'être synthétisée chimiquement, mais qu'elle peut être directement extraite du soja.

30. Par conséquent, il aurait été évident pour un homme du métier, sans aucune démarche inventive, de combiner D9 avec D10, et d'utiliser ainsi la daidzéine extraite du soja en tant que phyto-œstrogène dans un médicament pour traiter l'un des symptômes associés à la ménopause, à savoir l'ostéoporose.

31. En conclusion, l'objet de la revendication 1 est dénué d'activité inventive au regard de D9 combiné à D10.

Absence d'activité inventive au sens de l'article 56 CBE sur la base de D11 en combinaison avec D10

32. De D11 est connu le traitement et la prévention de l'ostéoporose par l'administration d'un médicament sous forme de dosage unitaire (une composition pharmaceutique) contenant une isoflavone, telle que la génistéine (R_1=OH et R_2=H dans la formule) ou la biochanine A (R_1=OCH_3 et R_2=H dans la formule). L'ostéoporose est connue pour être un symptôme associé à la ménopause (Cf. en particulier les raisons mentionnées au paragraphe 27 ci-dessus). La génistéine et la biochanine A sont par ailleurs connues comme présentant une activité oestrogénique.

33. Ainsi, l'utilisation selon la revendication 1 se distingue de D11 en ce que l'isoflavone est extraite de plantes de soja ou de trèfle.

34. Par conséquent, il aurait été évident pour un homme du métier de combiner D11 avec D10 pour les mêmes raisons que celles mentionnées au paragraphe 29 ci-dessus et de parvenir ainsi à l'objet de la revendication 1.

35. En conclusion, l'objet de la revendication 1 est dénué d'activité inventive au regard de D11 combiné à D10.

Revendication 2

Absence d'activité inventive au sens de l'article 56 CBE sur la base de l'un des documents choisis parmi D1, D2, D3, D4, D5, D6, D7, ou D8 en combinaison avec les connaissances générales de l'homme du métier

36. Le mode de réalisation couvert par la revendication dépendante 2 (ajout dans le médicament d'un excipient approprié sur le plan diététique) tombe dans le cadre des activités de routine de l'homme du métier.

37. Selon une pratique tout à fait habituelle, l'homme du métier aurait été incité à modifier très légèrement l'utilisation telle que décrite dans l'un des documents choisis parmi D1, D2,

D3, D4, D5, D6, D7, ou D8 à l'aide de simples mesures de routine pour parvenir à l'objet de la revendication 2.

38. Il est par ailleurs à noter que la revendication 2 dépendante ne semble résoudre aucun problème technique particulier.

39. Au vu de ce qui précède, la revendication 2 n'implique pas une activité inventive.

Absence d'activité inventive au sens de l'article 56 CBE sur la base de D9 en combinaison avec D10

40. En plus des caractéristiques mentionnées à l'alinéa 27 ci-dessus, il est également connu de D9 que le médicament comprend en outre un excipient approprié.

41. La même base d'argumentation qu'aux paragraphes 27 à 31 est reprise ici pour démontrer l'absence d'activité inventive de la revendication 2 au vu de D9 combiné D10.

Revendication 3

Absence de nouveauté au sens de l'article 54(2) CBE sur la base de chacun des documents choisis parmi D1, D2, D3, D4, D5, D6, D7, ou D8

42. En plus des caractéristiques mentionnées aux alinéas 1, 5, 8, 11, 14, 17, 20 et 23 ci-dessus, il est également connu de D1 ou D2 ou D3 ou D4 ou D5 ou D6 ou D7 ou D8 que le phyto-œstrogène isoflavone est extrait du soja.

43. Par conséquent, toutes les caractéristiques de la revendication 3 sont également connues de D1 ou D2 ou D3 ou D4 ou D5 ou D6 ou D7 ou D8, dénuant l'objet de cette revendication de nouveauté.

Absence d'activité inventive au sens de l'article 56 CBE sur la base de l'un des documents choisis parmi D1, D2, D3, D4, D5, D6, D7 ou D8, ou leurs combinaisons

44. Si, par impossible, la revendication 3 venait à être considérée comme nouvelle au vu de l'un des documents choisis parmi D1, D2, D3, D4, D5, D6, D7, ou D8, il y a alors lieu de considérer la revendication 3 comme n'impliquant pas d'activité inventive.

Absence d'activité inventive au sens de l'article 56 CBE sur la base de D9 en combinaison avec D10

45. La même base d'argumentation qu'aux paragraphes 27 à 31 est reprise ici pour démontrer l'absence d'activité inventive de la revendication 3 au vu de D9 combiné D10.

Absence d'activité inventive au sens de l'article 56 CBE sur la base de D11 en combinaison avec D10

46. La même base d'argumentation qu'aux paragraphes 32 à 35 est reprise ici pour démontrer l'absence d'activité inventive de la revendication 3 au vu de D11 combiné D10.

Revendication 4

Absence d'activité inventive au sens de l'article 56 CBE sur la base de l'un des documents choisis parmi D1, D2, D3, D4, D5, D6, D7, ou D8, en combinaison avec D12

47. Chacun des documents choisis parmi D1, D2, D3, D4, D5, D6, D7, ou D8 divulgue toutes les caractéristiques de la revendication 1 et de la revendication 3, pour les raisons mentionnés aux paragraphes 1 à 25 et 42 à 43 ci-dessus.

48. Ainsi, l'utilisation selon la revendication 4 se distingue de chacun des documents choisis parmi D1, D2, D3, D4, D5, D6, D7, ou D8 en ce que le phyto-œstrogène isoflavone est extrait d'hypocotyles de soja.

49. Or, D12 enseigne page 83, 4ème paragraphe, que les isoflavones peuvent être extraites d'hypocotyles de soja.

50. Par conséquent, il aurait été évident pour un homme du métier, sans aucune démarche inventive, de combiner chacun des documents choisis parmi D1, D2, D3, D4, D5, D6, D7, ou D8 avec D12, et de parvenir à l'objet de la revendication 4.

51. En conclusion, l'objet de la revendication 4 est dénué d'activité inventive au regard de l'un des documents choisis parmi D1, D2, D3, D4, D5, D6, D7, ou D8 en combinaison avec D12.

Revendication 5

Absence de nouveauté au sens de l'article 54(2) CBE sur la base de D2

52. En plus des caractéristiques mentionnées à l'alinéa 5 ci-dessus, il est également connu de D2 que le phyto-œstrogène isoflavone est extrait du trèfle.

53. Par conséquent, toutes les caractéristiques de la revendication 5 sont également connues de D2, dénuant l'objet de cette revendication de nouveauté.

Absence d'activité inventive au sens de l'article 56 CBE sur la base de D2

54. Si, par impossible, la revendication 5 venait à être considérée comme nouvelle au vu de D2, il y a alors lieu de considérer la revendication 5 comme n'impliquant pas d'activité inventive, pour les mêmes raisons que mentionné ci-dessus au paragraphe 26.

Absence d'activité inventive au sens de l'article 56 CBE sur la base de l'un des documents choisis parmi D1, D3, D4, D5, D6, D7 ou D8, en combinaison avec D12

55. Le mode de réalisation couvert par la revendication dépendante 5 (utilisation d'un phyto-œstrogène isoflavone extrait du trèfle) tombe dans le cadre des activités de routine de l'homme du métier, qui savait, à la date dont bénéficie l'objet de la revendication 5, à l'aide de ses connaissances générales (Cf. notamment D12), que les isoflavones phyto-œstrogènes peuvent être extraits du trèfle.

56. Selon une pratique tout à fait habituelle, l'homme du métier aurait été incité, en partant de l'un des documents choisis parmi D1, D3, D4, D5, D6, D7, ou D8, à utiliser des isoflavones extraits du trèfle, plutôt que du soja, pour parvenir à l'objet de la revendication 5.

57. Il est par ailleurs à noter que la revendication 5 dépendante ne semble résoudre aucun problème technique particulier.

58. Au vu de ce qui précède, la revendication 5 n'implique pas une activité inventive.

Revendication 6

Absence de nouveauté au sens de l'article 54(2) CBE sur la base de chacun des documents choisis parmi D2, D3, D4, D5, D6, D7, ou D8

59. En plus des caractéristiques mentionnées aux alinéas 5, 8, 11, 14, 17, 20 et 23 ci-dessus, il est également connu de D2 ou D3 ou D4 ou D5 ou D6 ou D7 ou D8 que la génistéine ou la daidzéine est une isoflavone phyto-œstrogène.

60. Par conséquent, toutes les caractéristiques de la revendication 6 sont également connues de D2 ou D3 ou D4 ou D5 ou D6 ou D7 ou D8, dénuant l'objet de cette revendication de nouveauté.

Absence d'activité inventive au sens de l'article 56 CBE sur la base de D1 en combinaison avec D10

61. Même si D1 ne mentionne pas la nature exacte des isoflavones utilisées, il ne fait aucun doute qu'un homme du métier savait, à la date dont bénéficie l'objet de la revendication 6, à l'aide de ses connaissances générales (Cf. en particulier D10) que la génistéine, la daidzéine, ou leurs dérivés sont des isoflavones du soja.

62. Au vu de ce qui précède, la revendication 6 n'implique pas une activité inventive, au vu de D1 combiné avec les connaissances générales de l'homme du métier illustrées par D10.

Absence d'activité inventive au sens de l'article 56 CBE sur la base de D9 en combinaison avec D10

63. En plus des caractéristiques mentionnées à l'alinéa 27 ci-dessus, il est également connu de D9 que l'isoflavone utilisée pour traiter l'ostéoporose est la daidzéine.

64. Par conséquent, l'objet de la revendication 6 est dénué d'activité inventive au regard de D9 combiné à D10 pour les mêmes raisons que mentionné ci-dessus aux alinéas 27 à 31.

Absence d'activité inventive au sens de l'article 56 CBE sur la base de D11 en combinaison avec D10

65. En plus des caractéristiques mentionnées à l'alinéa 32 ci-dessus, il est également connu de D11 que l'isoflavone utilisée pour traiter l'ostéoporose est la génistéine ou la biochanine A.

66. Par conséquent, l'objet de la revendication 6 est dénué d'activité inventive au regard de D11 combiné à D10 pour les mêmes raisons que mentionné ci-dessus aux alinéas 32 à 35.

Absence d'activité inventive au sens de l'article 56 CBE sur la base de l'un des documents choisis parmi D2, D3, D4, D5, D6, D7 ou D8, ou leurs combinaisons

67. Si, par impossible, la revendication 6 venait à être considérée comme nouvelle au vu de l'un des documents choisis parmi D2, D3, D4, D5, D6, D7, ou D8, il y a alors lieu de considérer la revendication 6 comme n'impliquant pas d'activité inventive pour les mêmes raisons que mentionné ci-dessus au paragraphe 26.

Revendication 7

Absence d'activité inventive au sens de l'article 56 CBE sur la base de D3

68. En plus des caractéristiques mentionnées à l'alinéa 8 ci-dessus, il est également connu de D3 que le ratio génistéine/daidzéine des urines des femmes japonaises est de 1,3 (Cf. tableau).

69. Comme D3 divulgue par ailleurs que l'excrétion des isoflavones dans les urines est directement liée à l'ingestion de produits à base de soja, le ratio génistéine/daidzéine des produits à base de soja ingérés est très certainement similaire au ratio mentionné ci-dessus, et à tout le moins tombe de manière évidente dans la plage 0,5 à 2.

70. Au vu de ce qui précède, la revendication 7 n'implique pas une activité inventive.

Absence d'activité inventive au sens de l'article 56 CBE sur la base de D7

71. En plus des caractéristiques mentionnées à l'alinéa 20 ci-dessus, il est également connu de D7 que le ratio génistéine/daidzéine des urines des souris est de 0,95 (Cf. tableau page 136, régime « soy+ »).

72. Comme D7 divulgue par ailleurs que l'excrétion des isoflavones dans les urines est directement liée à l'ingestion de produits à base de soja, le ratio génistéine/daidzéine des produits à base de soja ingérés est très certainement similaire au ratio mentionné ci-dessus, et à tout le moins tombe de manière évidente dans la plage 0,5 à 2.

73. Au vu de ce qui précède, la revendication 7 n'implique pas une activité inventive.

Absence d'activité inventive au sens de l'article 56 CBE sur la base de l'un des documents choisis parmi D1, D2, D4, D5, D6 ou D8 en combinaison avec les connaissances générales de l'homme du métier

74. Le mode de réalisation couvert par la revendication dépendante 7 (ratio particulier en isoflavones) tombe dans le cadre des activités de routine de l'homme du métier.

75. Selon une pratique tout à fait habituelle, l'homme du métier aurait été incité à préciser légèrement l'utilisation telle que décrite dans l'un des documents choisis parmi D1, D2, D4, D5, D6 ou D8 à l'aide de simples mesures de routine pour obtenir un ratio particulier en génistéine/daidzéine, et parvenir ainsi à l'objet de la revendication 7.

76. Il est par ailleurs à noter que la revendication 7 ne semble résoudre aucun problème technique particulier.

77. Au vu de ce qui précède, la revendication 7 n'implique pas une activité inventive.

Revendication 8

Absence d'activité inventive au sens de l'article 56 CBE sur la base de D9 en combinaison avec D10

78. En plus des caractéristiques mentionnées à l'alinéa 27 ci-dessus, il est également connu de D9 que l'isoflavone phyto-œstrogène est administrée à raison de 200 à 600 mg par jour sous forme de dose unitaire (Cf. page 7, lignes 31 à 32).

79. Par conséquent, la revendication 8 n'implique pas une activité inventive au vu de D9 combiné avec D10 pour les mêmes raisons que mentionné ci-dessus aux alinéas 27 à 31.

Absence d'activité inventive au sens de l'article 56 CBE sur la base de D11 en combinaison avec D10

80. En plus des caractéristiques mentionnées à l'alinéa 32 ci-dessus, il est également connu de D11 que l'isoflavone phyto-œstrogène est administrée à raison de 200 à 1000 mg par jour.

81. Par conséquent, la revendication 8 n'implique pas une activité inventive au vu de D11 combiné avec D10 pour les mêmes raisons que mentionné ci-dessus aux alinéas 32 à 35.

Absence d'activité inventive au sens de l'article 56 CBE sur la base de l'un des documents choisis parmi D1, D2, D3, D4, D5, D6, D7 ou D8 en combinaison avec les connaissances générales de l'homme du métier

82. Le mode de réalisation couvert par la revendication dépendante 8 (dose particulière en isoflavones) tombe dans le cadre des activités de routine de l'homme du métier.

83. Selon une pratique tout à fait habituelle, l'homme du métier aurait été incité à préciser légèrement l'utilisation telle que décrite dans l'un des documents choisis parmi D1, D2, D3, D4, D5, D6, D7 ou D8 à l'aide de simples mesures de routine pour obtenir une dose particulière en isoflavones, et parvenir ainsi à l'objet de la revendication 8.

84. Il est par ailleurs à noter que la revendication 8 ne semble résoudre aucun problème technique particulier.

85. Au vu de ce qui précède, la revendication 8 n'implique pas une activité inventive.

Revendication 9

Absence d'activité inventive au sens de l'article 56 CBE sur la base de l'un des documents choisis parmi D1, D2, D3, D4, D5, D6, D7 ou D8 en combinaison avec les connaissances générales de l'homme du métier

86. Le mode de réalisation couvert par la revendication dépendante 9 (durée d'administration particulière) tombe dans le cadre des activités de routine de l'homme du métier.

87. Selon une pratique tout à fait habituelle, l'homme du métier aurait été incité à préciser légèrement l'utilisation telle que décrite dans l'un des documents choisis parmi D1, D2, D3, D4, D5, D6, D7 ou D8 à l'aide de simples mesures de routine pour obtenir une durée de traitement particulière, et parvenir ainsi à l'objet de la revendication 9.

88. Il est par ailleurs à noter que la revendication 9 ne semble résoudre aucun problème technique particulier.

89. Au vu de ce qui précède, la revendication 9 n'implique pas une activité inventive.

Revendication 10

Absence de nouveauté au sens de l'article 54(2) CBE sur la base de chacun des documents choisis parmi D1, D4, D5, D6, ou D7

90. En plus des caractéristiques mentionnées aux alinéas 1, 11, 14, 17 et 20 ci-dessus, il est également connu de D1 ou D4 ou D5 ou D6 ou D7 que l'extrait phyto-œstrogène inclut des coumestanes, des lignanes et des flavones.

91. Par conséquent, toutes les caractéristiques de la revendication 10 sont également connues de D1 ou D4 ou D5 ou D6 ou D7, dénuant l'objet de cette revendication de nouveauté.

Absence d'activité inventive au sens de l'article 56 CBE sur la base de l'un des documents choisis parmi D1, D4, D5, D6 ou D7 ou leurs combinaisons

92. Si, par impossible, la revendication 10 venait à être considérée comme nouvelle au vu de l'un des documents choisis parmi D1, D4, D5, D6 ou D7, il y a alors lieu de considérer la revendication 10 comme n'impliquant pas d'activité inventive.

Absence d'activité inventive au sens de l'article 56 CBE sur la base de l'un des documents choisis parmi D2 ou D3 en combinaison avec D12

93. Même si D2 ou D3 ne mentionne pas explicitement que le soja ou le trèfle contient des coumestanes, des lignanes et des flavones, il ne fait aucun doute qu'un homme du métier savait, à la date dont bénéficie l'objet de la revendication 10, à l'aide de ses connaissances générales (Cf. en particulier D12) que l'extrait de soja ou de trèfle contient des coumestanes, des lignanes et des flavones.

94. Au vu de ce qui précède, la revendication 10 n'implique pas une activité inventive, au vu de D2 ou D3 combiné avec les connaissances générales de l'homme du métier illustrées par D12.

Revendication 11

Absence d'activité inventive au sens de l'article 56 CBE sur la base de D9 en combinaison avec D10

95. En plus des caractéristiques mentionnées à l'alinéa 27 ci-dessus, il est également connu de D9 que le médicament est administré sous forme de comprimé ou capsule (Cf. revendication 2, et page 7, lignes 34 à 35).

96. Par conséquent, la revendication 11 n'implique pas une activité inventive au vu de D9 combiné avec D10, pour les mêmes raisons que mentionné ci-dessus aux alinéas 27 à 31.

Absence d'activité inventive au sens de l'article 56 CBE sur la base de l'un des documents choisis parmi D1, D2, D3, D4, D5, D6, D7 ou D8 en combinaison avec les connaissances générales de l'homme du métier

97. Le mode de réalisation couvert par la revendication dépendante 11 (forme du dosage unitaire) tombe dans le cadre des activités de routine de l'homme du métier.

98. Selon une pratique tout à fait habituelle, l'homme du métier aurait été incité à préciser légèrement l'utilisation telle que décrite dans l'un des documents choisis parmi D1, D2, D3, D4, D5, D6, D7 ou D8 à l'aide de simples mesures de routine pour obtenir une forme de dosage unitaire particulière, et parvenir ainsi à l'objet de la revendication 11.

99. Il est par ailleurs à noter que la revendication 11 ne semble résoudre aucun problème technique particulier.

100. Au vu de ce qui précède, la revendication 11 n'implique pas une activité inventive.

Conclusion

Au vu de ce qui précède, le brevet opposé viole l'article 100(a) CBE, dans la mesure où toutes les revendications sont considérées comme non brevetables.

La requête d'une révocation totale du brevet est donc justifiée.

II. Article 100 (b)

Le brevet opposé ne divulgue pas l'invention d'une manière suffisamment claire et complète pour qu'elle puisse être exécutée par un homme du métier.

Le brevet ne fournit aucun exemple, ni aucune donnée expérimentale ou étude clinique permettant de montrer que l'extrait isoflavone phyto-œstrogène de soja ou de trèfle est en mesure de traiter efficacement le traitement des symptômes associés à la ménopause ou le cancer de la prostate.

Conclusion

Au vu de ce qui précède, il est requis la pleine révocation du brevet opposé en vertu de l'article 100(b) CBE.

Les motifs précités s'opposent au maintien du brevet attaqué.

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Paris, le 15 juin 2005

Fedex n°:7915 9315 4979

Opposition au brevet EP 656 786
Nos réf. : E21586 – FA/EL

Messieurs,

Vous voudrez bien trouver ci-joint un mémoire d'opposition que nous déposons au nom des sociétés Laboratoires ARKOPHARMA, PHYSCIENCE, LABORATOIRE OENOBIOL, LABORATOIRE LPH, TOURNAY BIOTECHNOLOGIES, LEA INSTITUT VITAL, LABORATOIRE THERAMEX, JUVA, NUTRINOV, NUTRITION ET SANTE, et par lequel nous demandons la révocation du brevet EP 656 786 dont le titulaire est la société NOVOGEN RESEARCH Pty Ltd et délivré le 15 septembre 2004, pour les motifs exposés dans ledit mémoire.

Nous joignons également la taxe d'opposition pour le paiement de laquelle nous vous remercions de débiter notre compte n° 28040004.

Nous vous prions d'agréer, Messieurs, l'expression de nos salutations distinguées.

Jacques WARCOIN

Pj : Mémoire d'opposition (2 ex)
Formulaire d'opposition (2 ex)
Bordereau de taxe (4 ex)
Documents D1 à D12 (2 ex)

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Catheterisation: your urethra in their hands

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The emphasis in undergraduate medical education is often on the theoretical aspects of medicine rather than the practical aspects. Practical procedures are commonly taught informally, the teaching being passed from one junior to the next.[1] The philosophy is of "See one, do one, teach one." Urethral catheterisation is a procedure that requires a certain amount of skill, knowledge, and experience and is not without complication,[2-4] yet it is usually delegated to the most junior and inexperienced medical staff, the junior house officers.

Subjects, methods, and results

To assess the level of competence at catheterisation among junior medical staff house officers at this hospital were interviewed with a structured questionnaire, covering three aspects of the procedure: the degree of undergraduate and postgraduate instruction, the practical and theoretical aspects of catheterisation, and, finally, problems and complications encountered.

Thirty junior house officers (graduates of five medical schools) were interviewed. Eighteen were male and 12 were female. The replies to the questionnaire showed that none of the interviewees had received any formal instruction regarding any aspect of urethral catheterisation as an undergraduate. Practical postgraduate instruction in 24 was limited to supervision of a single catheterisation, and four subjects were unsupervised. Although those interviewed had performed a mean of 28 (range 6-100) catheterisations in male patients, only four of them had catheterised female patients.

Despite the large number of procedures performed there was appreciable ignorance of the practical and theoretical aspects of catheterisation. Twenty five interviewees were unaware of the availability of short term and long term catheters or of the duration for which they may be safely left without being changed. Three interviewees simply used the catheter that was provided by the nursing staff, and one did not know that different sizes existed.

Twenty eight interviewees initially used force when meeting resistance to the passage of the catheter, and 13 stated that the development of fresh urethral bleeding would not deter them from a further attempt at catheterisation. Eighteen were happy to attempt catheterisation in a patient who had a known urethral stricture. Five interviewees were unaware of the difference between a phimosis and paraphimosis.

Despite the lack of formal tuition all had developed what seemed to be a satisfactory aseptic technique. None, however, was aware of the nature of the antiseptic fluid or the strength of the local anaesthetic gel, but simply used what was provided by the nursing staff.

Nineteen of the interviewees had encountered bleeding and six had had patients in whom a paraphimosis had developed after catheterisation. A particularly disturbing finding was that, although 14 interviewees had requested help from senior medical staff, seven were reluctant to seek advice, because of their impression that difficulties with catheterisation were not worthy of disturbing senior staff. Eight of the 12 female medical staff had encountered problems with male patients becoming sexually excited during the procedure.

Discussion

The results of our survey suggest that the technique of urethral catheterisation is poorly taught, and in the light of these results we are preparing a short teaching video to be shown to every house officer at the start of their preregistration post.

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Oestrogenic effects of plant foods in postmenopausal women

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Br Med J 1990;301:905-6

Crops grown as animal pasture are known to have oestrogenic activity,[1] and some foods contain potential oestrogenic analogues such as the isoflavonoids (isoflavones and coumestans), lignans, and resorcyclic acid lactones,[2] which may be activated or inactivated.[3] We studied the effect of three foods reported to induce vaginal oestrus in laboratory animals[4] in postmenopausal women not taking oestrogen replacement therapy.

Subjects, methods, and results

We studied 25 postmenopausal women who were non-smokers, in good general health, and taking no drugs known to affect oestrogen state (mean age 59 (range 51-70); body mass index 24·4 (range 18·7-31·6) kg/m^2; years after menopause 8·1 (range 1-20)). The protocol was a latin square design with a two week run in period and a six week experimental period. The women recorded their normal diet for 14 days and were asked to repeat the fortnightly diet throughout the study. During the experimental period the diet was

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supplemented with soya flour (45 g daily), red clover sprouts (10 g dry seed daily), and linseed (25 g daily), each for two weeks in turn. To check compliance the women returned residual food. Blood samples were taken weekly and lateral wall vaginal smears taken fortnightly and at follow up two and eight weeks after supplementation finished. Analysis was on intention to treat, but 23 women completed the study.

We examined the dependent variables vaginal cell maturation and serum concentrations of luteinising hormone and follicle stimulating hormone. The cumulative effects of the three foods at six weeks were compared with baseline by the paired *t* test, as were the residual effects, two and eight weeks after the last food supplement. We found significant differences in vaginal cytology after six weeks' supplementation ($p<0·01$, 95% confidence interval 6·0 to 17·6), which persisted for two weeks after treatment ($p<0·02$), but cytology returned to baseline after eight weeks (table).

Mean (SE) values for oestrogenic indicators in postmenopausal women consuming phyto-oestrogens

Week		Maturation value	Luteinising hormone (IU/l)	Follicle stimulating hormone (IU/l)
1			45·7 (3·1)	58·7 (2·9)
2		30·8 (4·5)	46·6 (3·4)	58·7 (3·0)
3			50·8 (8·5)	57·4 (2·9)
4		35·0 (5·1)	46·0 (3·6)	57·3 (2·9)
5	Food		46·2 (3·3)	57·7 (3·0)
6	supplementation	39·6 (5·3)	42·9 (3·2)	54·3 (2·9)
7			43·6 (3·3)	56·4 (2·8)
8		43·4 (3·6)	44·6 (3·3)	56·6 (2·4)
9			44·9 (3·5)	57·9 (2·8)
10		43·6 (4·7	44·9 (3·3)	57·5 (2·7)
16		33·7 (5·5)		

The maturation value significantly increased after soya flour ($p<0·05$) and linseed ($p<0·02$) but not after red clover sprouts ($p=0·11$).

All women had concentrations of follicle stimulating hormone and luteinising hormone greater than those in the premenopausal range of 2-8 IU/l and 6-13 IU/l respectively. There was a cumulative effect on serum concentrations of follicle stimulating hormone ($p<0·05$) but not on luteinising hormone over the six week supplementation period. Individual two week food supplements had no measurable effects on either hormone.

In seven women with the most pronounced changes in vaginal cytology we measured serum oestradiol concentrations weekly. Baseline concentrations were <70 pmol/l in all but one woman, who was retained as the study was based on intention to treat. There were no appreciable changes in body weight during the study.

Comment

We aimed to consider whether phyto-oestrogens were of consequence in human nutrition. Our study gives some indication of the recovery time from any possible effect of treatment and also provides further evidence of causality. Vaginal maturation is a sensitive and specific indicator of oestrogenicity. Follicle stimulating hormone is less sensitive to weak oestrogenic compounds such as phyto-oestrogens. Weak oestrogenic compounds may sometimes act as anti-oestrogens, which may affect their usefulness as sources of oestrogenic activity. Conversely, tamoxifen, an antioestrogen, can have oestrogenic effects on vaginal cytology.[5]

Patterns of food intake may modulate the severity of the menopause as it is an oestrogen deficiency state. Up to half of the diet of some populations may comprise foods containing phyto-oestrogens, whereas in our study such foods comprised only about 10% of energy intake for a fairly short time. Whether menopausal symptoms differ in such populations would be worth investigation.

We thank our statistical adviser, Steve Farrish, from the department of social and preventive medicine, Monash University.

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Inadvertent duplicate publication

Loop diathermy excision of the cervical transformation zone in patients with abnormal cervical smears

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The *BMJ* regrets that much of the material in the above article (30 June 1990, p 1690) was substantially the same as that published previously in *Contemporary Reviews in Obstetrics and Gynaecology* (Redman CWE, Buxton EJ, Cullimore J, Luesley DM. Loop diathermy excision of the cervical transformation zone in the management of cervical intraepithelial neoplasia. 1990;2:53-8). The authors did not tell us this when the article was submitted, their article did not contain any reference to the earlier paper, and all authors signed our copyright form, which states, among other things, that "papers are accepted on condition that they have not been published by any other journal."

We regret this inadvertent duplicate publication, for which the authors hold sole responsibility, and which is in violation of our Instructions to Authors and internationally agreed guidelines.

Correction

Incidence of peptic ulcer disease in Gothenburg, 1985

An editorial error occurred in this paper by Dr Ivi-Mai Schöt and others (1989;299:1132). The y axis of figure 1 should read 5, 10, 15, and 20 and not 0, 0·5, 1·0, 1·5, and 2·0 as published.

D2

Herbal Help to Avoid Menopause Symptoms

by Nancy Beckham

The aim of this article is to provide information on non-harmful ways of overcoming the problems of menopause.

The information given may also be applicable to women who already have osteoporosis or for younger women who have had their ovaries removed, however these two categories of women should seek professional guidance.

SOME STATISTICS

Twenty-five per cent of women in the 45–55 age range have no menopausal symptoms. Of the 75 per cent who have problems, the following is a breakdown of the symptoms:

Flushing and sweats	80%
Lethargy	70%
Nervous problems such as anxiety, depression, irritability	70%
Reduced sex drive	65%
Insomnia	60%

Other symptoms include hair and skin changes, poor memory and lack of concentration, headaches, dry vagina, pain during intercourse, loss of confidence, loss of femininity and urinary symptoms. Of course, not all of these are necessarily linked to low oestrogen levels and could be related to dietary and lifestyle factors and the 'normal' aging process. After middle age, men also find they have less energy, a lower sex drive and generally sleep less.

Osteoporosis is the most serious problem associated with menopause because as much as 50 per cent of total bone mass may be lost by the time a woman reaches 70 years of age, which means that bones can fracture easily and healing may be prolonged. This disease does not affect all regions – it is rare in African Negroes and there are areas where it affects more men than women. In Australia, it is estimated that about 25 per cent of post-menopausal women have osteoporosis. I will deal with this in detail next issue.

What happens when the ovaries stop functioning?

The major factor is the lowered production of oestrogen. However, this hormone can be produced in other glands, such as the adrenals, but obviously, in many women, this does not occur quickly enough or in sufficient quantities. Basically, the hormonal system works on a feedback system; when the circulating levels are high, a chemical 'messenger' instructs our endocrine system not to produce any more of that particular hormone. Obviously, if we flood our system with a hormonal drug, the messages to our endocrine system will be to stop production. This may explain why some women do not menstruate for varying periods when they stop taking the Pill.

Most menopausal-age women will need to give their bodies as much assistance as possible so that sufficient oestrogen is produced to offset flushing and other symptoms. In nearly every case, this apparently happens over a period of time as the obvious symptoms gradually lessen and disappear. This added function of the adrenals may partly explain why some women have difficulty handling stress at menopause.

The controversial topic of hormonal replacement therapy will be discussed in detail later but in view of this feedback mechanism, it may not be wise to completely dampen the corrective biological function which already exists.

I am not suggesting that we can avoid the inevitability of aging, but I can't accept that whatever power 'designed' us also programmed that we were predestined to suffer a range of serious problems after middle age. We must be doing something wrong or there must be non-harmful methods of preventing the symptoms.

OESTROGENS IN FOODS AND HERBS AND HOW TO USE THEM

Since the 1920s over 50 different species of plant have been found to contain oestrogenic substances. Most of the published research papers relate to the effects on animals, particularly in respect of clovers and alfalfa (lucerne) causing infertility in farm animals. It so happens that a number of these plants have been used by herbalists over the centuries and this 'tested' use on humans has verified the hormonal effect. The tiny quantities of oestrogens in plants are extremely weak compared to pharmaceutical hormones but many women alleviate symptoms through sensible dietary changes.

Some of these oestrogen-containing plants are:

Alfalfa

The sprouts are particularly recommended as they have the added advantage of being very low in calories, readily available in shops or you can make your own, palatable in salads or sandwiches, mildly alkaline and rich in nutrients, especially calcium and potassium.

Alfalfa sprouts are somewhat controversial at the moment as the Gerson Institute in Mexico has reported that they suppress the immune system and aggravate conditions such as rheumatoid arthritis and systemic lupus erythematosus (SLE). The origin of this report was that two women had seemingly reactivated SLE following the ingestion of 10 and 15 alfalfa tablets per day. A particular constituent, L-canavanine, was extracted from alfalfa and when this isolated extract was given to susceptible animals, SLE was reactivated.

My own view is that, as SLE is a condition which has relative periods of aggravation and remission, it would be difficult to 'blame' one particular dietary item. Over 25 pharmaceuticals exacerbate the disease, isolated extracts of plants are in the nature of drugs and one would therefore expect side-effects and, most importantly, if this type of criterion were applied to almost any edible food, there would be very little left for us to eat. However, it may be prudent for sufferers of SLE to avoid alfalfa, all sprouted seeds and legumes, such as lentils, because these also contain the suspected irritant.

Red Clover

This is commonly sold as a herbal tea but I suggest you buy the seeds and sprout them. Please do not pick the clover yourself because the medicinal species, called *Trifolium pratense*, is difficult to distinguish from some non-edible clovers. Red clover is also used by professional herbalists for skin and respiratory conditions.

Sage

The common garden sage or red sage is used. It is better to grow your own but sage can be difficult to cultivate, mainly because it prefers a light, well-drained soil.

Sage has been used for centuries for excess sweating and heat, and scientific research has confirmed its oestrogen content. The best way to prepare it as a remedy for flushing is to soak two

tablespoons of finely chopped fresh leaves (or one tablespoon dried) in 500 mL tepid water with the juice of a lemon. Leave it stand in a covered jar overnight. Strain and keep in the fridge. In severe cases you would drink the whole quantity throughout the day; where the symptoms are relatively minor, then the 500 mL could be spread over two or three days. To make it more palatable, you could mix it with a fruit or vegetable juice, or add in some crushed fennel or aniseed. The high dose may need to be used for up to four weeks, then it could be gradually reduced to a cup per day.

The old sage you have had in your cupboard since two Christmases ago probably no longer retains any therapeutic properties; good-quality dried sage will still have a reasonably good colour and its characteristic strong odour.

Parsley

This common culinary herb has oestrogen-like activity and I would suggest a handful per day; it may not be wise to use larger quantities because of the myristicin and apiol content.

Aniseed

Use the crushed seeds as a herbal tea or in cooking, for example in home-made bread. The seeds could also be added to apple cider vinegar and used in salad dressings. Finely chopped fresh leaves can be added to salads, steamed vegetables and soups. Aniseed is also helpful for minor digestive problems and coughs.

Fennel

The seeds and finely chopped fresh leaves can be used in a similar way to aniseed. There is one species of fennel (Florence) which develops a bulb-like base and this may be used like celery or lightly steamed. Some greengrocers call it aniseed root. Wild fennel is a common weed and, although the seeds and leaves could be used, this plant is often contaminated with environmental pollutants.

Similar culinary herbs, such as dill and caraway, probably contain mild oestrogen-like substances.

Hops

Some health-food stores sell dried hops. It is somewhat bitter, which may also stimulate the digestive function, but the tea should be made quite weak otherwise it is not very palatable. An important feature of hops is that it has a sedative function and for this reason herbal extracts of hops are not used by professional herbalists where there is depression. Many people find that hops helps with insomnia – a herbal pillow using dried hops can be quite beneficial.

The hormonal content of hops has been verified; females harvesting it have altered menstrual periods solely from external contact. I am not sure whether or not beer, after all the processing, would retain any oestrogenic properties.

Soya Beans

Sprouts are the best way to have these, particularly as the sprouting dramatically increases the oestrogen content. However, they are quite difficult to sprout because they go mouldy and smelly if not washed and drained thoroughly and often. They are amazingly tasty but wait until you have learned to sprout alfalfa and mung beans before trying them. I add soya bean sprouts to salads or use them to thicken soups and casseroles.

Dried soya beans need to be soaked and cooked for a long time and they are probably best used in soups and casseroles but there are many ways of preparing them to make them more appetising. They are cheap, an excellent protein when combined with a grain and have other benefits, such as being protective against atherosclerosis.

If you don't normally eat dried beans then you must start with small quantities, soaked overnight and very well cooked, otherwise you will probably have severe abdominal colic and flatulence. This is partly because certain enzymes have to be activated to handle such foods and your digestive system needs time to adjust.

Soya beans are also a leguminous plant so, theoretically, could have the same cautions as indicated under alfalfa.

Dried red beans and common green beans are also mildly oestrogenic so could be included in the diet on a regular basis.

There is some evidence that all young sprouts, including sprouted grains and legumes, have oestrogenic properties and, as sprouts are cheap, pesticide and chemical free, rich in nutrients and low in calories, I recommend that you learn how to do your own and have at least one cup per day if you are a menopause-age female. You can buy small paperback books giving you basic instructions for sprouting and use jars, so the starting equipment is not expensive.

Some words of warning: When using seeds to sprout, never use those that are intended for agricultural purposes because they would have been treated with fungicides or other chemicals which are potentially dangerous.

Fenugreek

Contains precursors of progesterone, another female hormone commonly deficient in menopausal women. Unfortunately, the curry-like smell is readily excreted through the skin but this is not so noticeable if the seeds are sprouted.

There are other herbal and naturopathic remedies for menopausal problems but these are not normally available at retail outlets so you would need to visit a practitioner to obtain these. As with most health problems, there are mild symptoms which require no treatment or simple home remedies; then there are other instances where professional naturopathic advice is helpful and appropriate; and, finally, there are severe cases which require medical diagnosis and treatment.

OTHER SUGGESTIONS

Potassium sulphate, used in the form of tissue salts, may be helpful for flushing. Use the dosage on the label, but take double the dose for the first week.

Vitamin E has also alleviated some cases of flushing; furthermore, a study on rats showed that a vitamin E deficiency leads to lower bone weight. As this vitamin has benefits to the cardiovascular system, a supplement of 500 iμ per day would do no harm and may give marked benefits.

Cigarette smoking tends to bring on early menopause and is not recommended for this and other well-publicised reasons.

Low-calorie diets are not recommended for a number of reasons which are given later, but one important factor is that fat cells are able to convert hormones from the adrenal glands into oestrogen. Although modern women, including myself, don't want or need to be obese, it may be that 'nature' intended us to carry more weight as we age.

Readers may be interested in a few snippets from some of the research material which I have collected:

Journal of Food Protection, Vol. 42, July 1979, states that 'human exposure to dietary oestrogens is below physiological levels ... but the possibility of metabolic alterations to more or less active forms should not be ignored since effects of this kind have been demonstrated in experimental animals.'

Oestrogenic Constituents of Forage Plants, E.M. Bickoff, Review Series 1/1968, published by the Commonwealth Bureau of Pastures and Field Crops, Hurley, Berkshire, reports that 'the classical infertility syndrome in ewes is associated with the cumulative effects of exposure to oestrogenic feeding for six months or longer, but short-term exposure has also caused reproductive disturbances.'

The effect of oestrogenic plant substances is judged by changes in the anatomy of animals, for example increased uterine and ovarian weight, teat length and thicker vaginal skin.

A particularly interesting piece of research has shown that genistein, a weak plant oestrogen, is able to displace oestradiol from receptors in the tissues which could explain why some herbal

(continued on next page)

Herbal Help to Avoid Menopause Symptoms

remedies are traditionally used for 'balancing' hormones.

Both the liver and the kidneys have a capacity for converting and deactivating different types of oestrogens; there are also other regulatory mechanisms, such as the prostaglandins.

HORMONE REPLACEMENT THERAPY

Although mainstream medical opinion supports hormone replacement therapy, it is somewhat controversial. Few disagree with the fact that it prevents the worsening of osteoporosis in postmenopausal females but the main disadvantage is in the potential side-effects. The current scientific thinking is that if both oestrogen and progestogen are taken together there is less risk of cancer. I am using Depo-Provera and Premarin as examples because a lady I saw recently had been prescribed these for menopausal flushing and she had written to the two manufacturers for information. The manufacturer of Premarin sent back 22 pages of reports and a book, all giving glowing testimonials and other information but only a few fragments about risks. The manufacturer of Depo-Provera sent a copy of the official package insert, with all the contraindications and side-effects, along with a letter which stated that 'as adjunct to cyclic oestrogen therapy (including Premarin) Depo-Provera is not recommended but it is still definitely used by medical practitioners'.

Depo-Provera

The contraindications and warnings for this drug include thromboembolic disorders (clotting), cerebral apoplexy (stroke), impaired liver function, undiagnosed vaginal bleeding and cerebrovascular (heart and circulatory) disorders. 'In cases of partial or complete loss of vision, sudden onset of proptosis (displacement of an organ), double vision, migraine associated with retinal vascular lesions, medication should be withdrawn.' The drug caused malignant breast nodules in animals. Other problems include fluid retention, breakthrough bleeding and depression. It is not approved for contraception because of unresolved questions relating to its safety for this purpose. Clinically, it is said that the drug is well tolerated although animal studies show that it possesses adrenocorticoid activity and female masculinisation.

Premarin

This drug is 'probably effective for oestrogen deficiency-induced osteoporosis only when used in conjunction with other important measures such as diet, calcium, physiotherapy and good general health-promoting measures'. The contraindications and cautions include impaired liver function, breast cancer (with some exceptions), thromboembolic disorders, undiagnosed abnormal genital bleeding and pregnancy. It should not be given to women with recurrent chronic mastitis and abnormal mammograms. It should only be prescribed following a complete breast and pelvis examination. Because the body produces variable amounts of oestrogen, relative overdosage may occur which could lead to uterine bleeding, painful, swollen breasts and fluid retention. Drug oestrogens need to be used with care in cases of epilepsy, migraine, asthma, heart or kidney disease. Side-effects include nausea, abdominal cramps and bloating, breast tenderness, changes in body weight, allergic rash and gallbladder complications.

When the two are prescribed together, there is often a monthly bleed.

If you are one of those people who believe that 'it would not be allowed by the government if there were risks involved', I suggest you read the information for yourself in *Mims Annual* which is available at most libraries.

A report in the *New England Journal of Medicine*, 19 June 1986, states that 'oral administration of oestrogens is inefficient, produces a non-physiologic pattern of breakdown products and increases undesirable levels of certain liver proteins'. The article examines the use of transdermal oestrogen therapy (skin patches) which would provide levels equivalent to those produced naturally. However, according to *Modern Medicine in Australia*, August 1986, transdermal oestrogen does not prevent osteoporosis.

Mainstream medical reports recommend oestrogen, used in conjunction with progesterone, as being the most effective therapy for preventing fractures, and a number of international experts suggest that all women should be considered probable victims and that hormone replacement therapy should begin soon after menopause in women, unless there are specific contraindications. The reasons for this are that at this stage there are no practical methods of clearly preselecting those at risk and tests show that it is the only therapy that clearly prevents further bone deterioration.

The critics point out that although adding progesterone to the therapy probably reduces the risk of endometrial cancer, there is insufficient data about the long-term safety or benefits – it only treats the symptoms and is unlikely to help the body re-establish its own state of internal harmony.

A pamphlet issued by the NSW Department of Health states that you should 'think carefully about whether or not you want hormone replacement therapy'. Some of my patients have understood from their medical consultations that hormone replacement therapy prevents cancer and cardiovascular disease, which is not true. Patients who are under this impression should discuss this matter with their practitioners.

The fact that a substance can prevent further bone breakdown does not necessarily mean that lack of it caused the problem, just as Valium helps you sleep but lack of it did not cause your insomnia.

In any event, most experts agree that there are clearly individuals who should not undertake hormonal replacement therapy and such people can use the non-harmful suggestions in this article.

No one enjoys being wrinkled and cranky but it is generally conceded that hormonal replacement therapy is not appropriate for cosmetic and emotional purposes.

Next issue: Osteoporosis and calcium requirements.

Nancy Beckham is the author of The Family Guide to Natural Therapies, *Greenhouse Publications, recommended retail price $24.95.*

(D3)

in newborn babies support an essential role for n-3 fatty acids in retinal development.[5]

The DHA content of erythrocytes is small compared with that of grey-matter, but the fatty acid composition of the erythrocyte membranes may indicate the fatty acid status of neural and perhaps other membranes. During the period of most rapid DHA accumulation in the developing rat, diet-induced changes in neural DHA are reflected in red blood cell DHA.[6]

Dietary n-3 fatty acids can also modify endogenous prostaglandin/ production and perhaps by this means influence uterine prostaglandins and gestation time. In the Faroe Islands, where birthweights are amongst the highest in the world with long gestation periods and rapid fetal growth, the intake of marine fat rich in n-3 fatty acids is high and erythrocyte DHA values in pregnant women were found to be almost twice those in normal individuals in other countries.[7]

The lower erythrocyte DHA found in patients on epoetin could be due to an increased requirement for this n-3 fatty acid as a result of increased red cell production, and this implies a deficiency of or a rate-limited production of DHA. However, plasma DHA values were not low, which raises the possibility of a defect of incorporation of this fatty acid into the membrane in patients on haemodialysis.

A low membrane DHA probably has little effect on red cell function and may be of minor importance in adults, although it is of interest that visual hallucinations have been described in patients on epoetin.[8] Unlike the adult, the fetus requires DHA in quantity for its developing nervous system, and haemodialysed patients do occasionally become pregnant. For reasons not fully understood, pregnancy in uraemia is associated with a high risk of premature labour and retarded fetal growth.[9] A lack of DHA would be detrimental to the fetus, and our results indicate that in a uraemic pregnant woman on haemodialysis, low quantities of membrane DHA could be one of the hazards to which the fetus is exposed. Because epoetin gives rise to even lower membrane DHA content, its use could increase the risk to the fetus: n-3 fatty acid dietary supplements are indicated.

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Dietary phyto-oestrogens and the menopause in Japan

SIR,—Lock, in an article on the menopause,[1] has discussed differences between Japanese women and women in western societies. Japanese women have a much lower frequency of hot flushes than women in Canada. Lock concluded that "cultural indifference to the hot flush in Japan" was unlikely to account fully for these findings.

Recently our Helsinki group studied, in collaboration with Japanese scientists, the diet and phyto-oestrogen excretion in

URINARY EXCRETION OF ISOFLAVONOID PHYTO-OESTROGENS AND ENDOGENOUS OESTROGENS IN JAPANESE OR ORIENTAL WOMEN, AND IN AMERICAN AND FINNISH OMNIVOROUS WOMEN

Urinary isoflavonoid or oestrogen	Japanese/ Oriental	American	Finnish
Genistein,	3440 (n = 3)*	..	32·1 (n = 12)
Daidzein	2600 (n = 10)*	216 (n = 21)	40·5 (n = 12)
Equol	2600 (n = 10)*	62·8 (n = 21)	44·2 (n = 12)
Oestrone (postmenopausal)	4·48 (n = 9)†	..	4·48 (n = 10)
Oestradiol (postmenopausal)	0·76 (n = 9)†	..	0·94 (n = 10)
Oestriol (postmenopausal)	4·48 (n = 9)†	..	1·41 (n = 10)

All assays by gas chromatography/mass spectrometry in selected ion-monitoring mode with deuterated internal standards.[2] Women collected two to four 72 h urine samples 3–6 months apart and values are thus means of urinary excretion in individual subjects over 6–12 days. Results as geometric means in nmol/24 h
*Values from ref 2.
†Oriental postmenopausal women (recent emigrants to Hawaii). Same women as in ref 7, but oestrogens measured by new technique.[3]

Japanese women and men, and in a few children.[2] The women's mean age was 50·4 (SD 18·0) years and they were all from a small village south of Kyoto and consumed a traditional Japanese low-fat diet. We studied a group of three men, three women, and three children living in Kyoto and consuming the traditional diet, and in this group we measured the isoflavonoid genistein.[3] We found a very high excretion of phyto-oestrogens in urine. The mean values were almost identical in the two groups and especially high excretion was found for genistein (maximum 15·5 μmol per 24 h in a man) and two other isoflavonoids, daidzein and equol (table). All these compounds bind to oestrogen receptors and have weak oestrogenic activity.[4] The excretion of the isoflavonoids in urine of the Japanese women was much higher than in American and Finnish women (table) (ref 4 and unpublished data) and as high in children as in middle-aged and old people. These compounds were excreted in 100-fold to 1000-fold higher amounts than those of endogenous oestrogens in normal omnivorous women consuming a western or oriental diet (table).

The excretion of the isoflavonoids in urine was associated with intake of soy products such as *tofu, miso, aburage, atsuage, kori-dofu,* soybeans, and boiled beans. All isoflavonoids are weak oestrogens and such high amounts could have biological effects, especially in postmenopausal women with low oestrogen levels. High levels of isoflavonoid phyto-oestrogens may partly explain why hot flushes and other menopausal symptoms are so infrequent in Japanese women.

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Western diet and Western diseases: some hormonal and biochemical mechanisms and associations

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Breast cancer, prostate cancer, coronary heart disease and colon cancer belong to the so-called Western diseases and a general opinion is that diet is a significant or even the main factor increasing incidence and mortality of these diseases in the Western world. This review describes studies carried out in this department for about 10 years, many in collaboration with scientists abroad, and with the aim to clarify some of the connections between the diet and sex hormone, lipid and bile acid metabolism. A Western-type diet elevates plasma levels of sex hormones and decreases the sex hormone binding globulin concentration, increasing the bioavailability of these steroids. The same diet results in low formation of mammalian lignans and isoflavonic phytoestrogens. These diphenolic compounds seem to affect hormone metabolism and production and cancer cell growth by many different mechanisms making them candidates for a role as cancer protective substances. The precursors of these diphenols are to be found in fiber-rich unrefined grain products, various seeds, beans and probably also in pulses, peas and berries. Some types of fiber seem to influence sex hormone and bile acid metabolism mainly by partial interruption of the enterohepatic circulation, by alteration of intestinal metabolism and by increasing fecal excretion of these compounds. The sex hormone pattern found in connection with a Western-type diet is prevailing in the breast cancer patients, but is only partly a result of the diet.

Key words: breast cancer, prostate cancer, colon cancer, coronary heart disease, diet, fiber, lignans, isoflavones, estrogens, androgens, sex hormone binding globulin, dihydrotestosterone, bile acids, feces

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Breast cancer (BC), prostate cancer (PC) and endometrial cancer (EC) belong to the group of hormone-dependent cancers which in addition to colon cancer (CC), coronary heart disease (CHD) and some other diseases are called Western diseases because their incidence and mortality are high in the Western world compared to countries in Asia and South and East Europe [1–3]. In migrant studies an increased risk for Western diseases has been found to be related to a change towards a Westernized diet [4–9]. Migrants from Asia, Africa or East Europe to U.S.A. or Australia have

originally consumed a low-fat vegetarian or semi-vegetarian diet containing large amounts of unrefined carbohydrates. Most of these migrants and their children rapidly adopt a diet rich in calories, fat and proteins and low in complex carbohydrates and fiber [10] and their hormone [11, 12] and lipid levels change towards a Western pattern, increasing the risk for hormone-dependent cancer and CHD. Interestingly, migrants from a high risk colon cancer area (Scotland) to Australia experience a reduced risk for colon cancer [9].

Furthermore Hill et al. [13] postulated that a Western-type diet increases the concentration and metabolism of fecal bile acids (FBA) and neutral sterols (FNS), increasing the risk for CC. In the majority of the population studies carried out this hypothesis has rendered support with regard to the concentration but not with regard to the metabolism of FBA and FNS [review in 14]. On the other hand many animal experiments and *in vitro* tests have shown that free bile acids are cocarcinogenic or comutagenic [15, 16] but that the aminoconjugated bile acids may be inactive [16] in this regard [reviews in 14, 17]. It is believed that secondary bile acids are more toxic than primary ones and that a high litocholic acid (LCA) to deoxycholic acid (DCA) ratio is a CC risk factor [18–20].

Because of the obvious relationship between Western diet and Western diseases it has been postulated that this type of diet by some biochemical or other mechanisms may alter hormone production, metabolism or action at the cellular level increasing the risk for hormone-dependent cancer. Furthermore it has been suggested that the dietary composition may influence transit time of the intestinal content, fecal bulk and intestinal microflora and its environment causing alterations in concentration and metabolism of hormonal steroids, bile acids, neutral sterols, carcinogens and procarcinogens increasing the risk of CC and BC. Particularly in women, who have a much higher incidence of hormone-dependent cancer than men, diet has been suggested to be the main single determinant in the etiology of these cancers.

It is, however, very difficult to separate the effects of various single macro- or micronutritients on any biochemical event or steroid hormone or bile acid pattern or level. This is not only due to difficulties in the accurate recording of the diet, but also to the great variability in dietary intake during different seasons and even different parts of the week and the variability of hormone and steroid levels, particularly in women. Special efforts have to be made to standardize the conditions for sampling and to use reliable hormone assay methods and the recording of the diet must be carried out during sufficiently long time [12].

TABLE I. Abbreviations and trivial names of steroids and other abbreviations used in the text.

A	Androstenedione
BC	Breast cancer
CC	Colon cancer
CHD	Coronary heart disease
DHEAS	Dehydroepiandrosterone sulfate
Da	Daidzein
DCA	Deoxycholic acid
5α-DHT	5α-Dihydrotestosterone
EC	Endometrial cancer
End	Enterodiol
Enl	Enterolactone
E2	Estradiol
E3	Estriol
E1	Estrone
E1S	Estrone sulfate
Eq	Equol
FBA	Fecal bile acids
FNS	Fecal neutral sterols
For	Formononetin
FE2	Free estradiol
FT	Free testosterone
Gen	Genistein
2-OHE1	2-Hydroxyestrone
4-OHE1	4-Hydroxyestrone
%FT	Percentage free testosterone
%FE2	Percentage free estradiol
PC	Prostate cancer
LCA	Litocholic acid
LH	Luteinizing hormone
Mat	Matairesinol
SHBG	Sex hormone binding globulin
T	Testosterone

The following review will summarize and discuss results of our studies on the connection between diet and Western diseases. Many of these investigations are the result of collaborations with scientists abroad and some results discussed have not yet been published. The review will deal with some newly discovered mechanisms of dietary effects on sex hormone and intestinal bile acid metabo-

lism and in addition with some interesting associations between the various diseases. Further support for the previously proposed extension [21] of the "fiber hypothesis" of Burkitt & Trowell [see 10] has now been obtained and will be discussed including not only BC and CC but also other Western diseases.

EFFECT OF VARIOUS MACRONUTRITIENTS ON SEX HORMONE METABOLISM

Effect of fiber

The development of a radioimmunological chromatographic method for the assay of the very low amounts of estrogens present in feces of men and nonpregnant women [22] made it possible for the first time to obtain a complete view of the effect of diet on the enterohepatic circulation of estrogens in man.

A high intake of fiber in premenopausal women increases fecal wet and dry weight, which correlates positively with all three unconjugated estrogens and total estrogens in feces [23]. In the same study also postmenopausal women were investigated (H. Adlercreutz, E. Hämäläinen, S.L. Gorbach, B.R. Goldin, J.T. Dwyer, M.N. Woods, unpublished results) and the same results were found. Furthermore, in the postmenopausal women we found positive associations between total fiber and grain fiber intake, and fecal estrone (E1) and estradiol (E2) excretion (list of abbreviations in Table I). Fat intake on the other hand seems to have a negative association with fecal excretion of estrogens [24] and therefore the dietary fat/fiber ratio of the postmenopausal women living in Boston shows highly significant negative correlation with fecal estrogen excretion (above-mentioned unpublished study). It is suggested that the dietary fat/fiber ratio determines the degree of interruption of the enterohepatric circulation of steroids, but the type of fiber plays also a significant role (see below).

In premenopausal women fecal weight and fecal estrogen excretion was found to correlate negatively with urinary estrogen excretion [23]. Particularly important was the observation of a negative correlation between fecal estriol (E3) and urinary E3-3-glucuronide (E3–3G) excretion. Urinary E3-3G is a specific metabolite of the intestinal mucosal cell and the end-product of estrogen metabolism and therefore a good indicator of the extent of the enterohepatic circulation of estrogens, particularly of E3 and other 16-hydroxylated and polar estrogens in man [25]. In a study carried out in Helsinki in premenopausal women it was found that total fiber intake and grain fiber intake/kg body weight were negatively associated with the excretion of 10 of the 13 estrogens measured in urine [26].

Fecal estrogen excretion shows a negative association with plasma E1 and E2 [23] and later on a direct negative correlation between total fiber intake and plasma E1 and E2 [24] and estrone sulfate (E1S) [27] could be observed in young women. Similar findings in men have been reported, but in addition to the negative correlation between crude fiber intake and plasma E2, higher fiber intake is associated with lower plasma testosterone (T) levels [28–30]. The reason for reduced intestinal reabsorption and increased elimination of estrogens by the fecal route in subjects consuming much fiber seems to be the larger fecal bulk and decreased concentration of intestinal β-glucuronidase [21, 23, 25]. The latter phenomenon reduces hydrolysis of the biliary steroid conjugates, an event necessary for their reabsorption. Some fibers have also the property of binding sex hormones, particularly non-polar estrogens [31, 32].

Preliminary results in the large study in Helsinki, called the "Finlandia study" revealed significant positive correlations between intake of total fiber, vegetable fiber and fiber from fruits and berries and plasma sex hormone binding globulin (SHBG) and negative associations between the intake of the same fibers and plasma % free estradiol (%FE2). Furthermore, total fiber, grain fiber and vegetable fiber intake correlated negatively with plasma % free testosterone (%FT) [33, 34]. The new results obtained in in postmenopausal Boston women [23, 24] agree well with the above-cited publications in that significant negative correlations were found between intake of total fiber, grain fiber and non-grain fiber and plasma androstenedione (A), T, FT [35] and E1. In addition intake of fruit and vegetable fiber and grain calories correlated negative-

ly with plasma E1 [estrogen results unpublished, see 27].

It may be concluded that high fiber intake is associated with low levels of sex hormones in plasma, high SHBG and low %FE2 and %FT causing a reduction in the bioavailability of the hormones, which theoretically would reduce the risk of hormone-dependent cancer. The proposed mechanisms involved in changing the SHBG level will be discussed in the sections on dietary protein, and lignans and isoflavonic phytoestrogens.

Effect of protein

Most of the studies on the effect of protein intake on hormone metabolism have been carried out by altering the protein/carbohydrate ratio of the diet. Using this technique it was found that a high dietary protein/carbohydrate ratio decreases the plasma level of SHBG and T and that a low ratio has the opposite effect [36, 37]. Furthermore a high protein diet considerably diminished 4-ene-5α-reduction of T and enhanced 2-hydroxylation of E2 [38, 39]. By measuring the estrogen profile in urine by capillary GC-MS in premenopausal women [40, 41] we could recently confirm that a high dietary protein/carbohydrate ratio results in high urinary excretion of catecholestrogens. A new finding was that the dietary protein/carbohydrate ratio is highly significantly and positively associated with the urinary 2-OH-E1/4-OH-E1 ratio. Furthermore the lowest mean ratio (= 3.6) was found in vegetarians, followed by the omnivores (= 4.3) and the highest was found in the BC patients (= 7.1) (BC *vs.* vegetarians $p < 0.005$; BC *vs.* omnivores $p < 0.02$), who had the highest dietary protein/carbohydrate ratio due to low grain intake. It may be mentioned that this ratio was recently found to be 2.0 in the same Oriental migrant women in Hawaii [42], which were previously studied by us [24].

Effect of carbohydrates

In the above section the effect of changes in the dietary protein/carbohydrate ratio was discussed. Some further information as to the possible effect of carbohydrates on sex hormone metabolism derives from studies in which dietary intake of various macro- and micronutritients were correlated with plasma and urinary hormone levels.

Recently we found that postmenopausal women living in Boston showed significant negative associations between carbohydrate intake and plasma T, E1 and E2 [35,43]. Furthermore in the same study the intake of grain calories showed negative correlations with plasma A, T, DHEAS, and E1. The intake of carbohydrates also showed a weak but significant positive correlation with fecal E1 excretion (estrogen results unpublished).

In the corresponding Finnish study in 33 premenopausal women [40–42], studied twice during a year, we found some other interesting correlations between carbohydrates and sex hormones. Urinary 2-OH-E1/4-OH-E1 ratio correlated positively with protein/carbohydrate ratio of the diet and negatively with carbohydrate, starch, total fiber and grain fiber intake. Urinary 4-hydroxyestrone excretion correlated positively with total and grain fiber intake and plasma SHBG and negatively with %FE2 and %FT. Starch intake was negatively associated with urinary E3-3-glucuronide, the specific marker of the enterohepatic circulation of estrogens, suggesting partial interruption of this circulation in subjects with high starch intake. Carbohydrate intake was negatively associated with plasma E1S, the mean level of which was highest in the BC group. Plasma DHEAS on the other hand was strongly positively associated with plasma E1S, and less strongly with %FE2 and negatively associated with urinary 16-hydroxylated estrogens and enterolactone (Enl) [27]. Enl mainly derives from precursors in grain and its urinary excretion reflects both the intake of fiber in general [44] and whole-grain products in particular. The results indicate that it is difficult to separate the effect on hormone metabolism of complex carbohydrates from that of fiber.

Effect of fat

Oriental women living in East Asia and at low risk for BC consume a very low-fat diet (usually < 20 % of calories). Studies on the urinary excretion of E1, E2 and E3 have shown that they excrete lower amounts of E1 and E2 and similar amounts of E3

compared to women in Western countries [24, 45, 46]. In other studies in vegetarians living in Western societies the picture has not been so clear, but there has been a trend towards lower urinary E1 and E2 values and similar or slightly higher E3 values in the vegetarians [47, 48]. Thus a vegetarian or semivegetarian diet seems to be associated with relatively high E3 formation. The simultaneously higher fecal excretion of E3, however, reduces urinary E3 levels leading to varying quantitative results for E3 in urine, depending mainly on the nature of the fiber in the food and the quantity of both dietary fiber and fat. Simultaneously there seems to be a reduction in the relative concentration of 2-hydroxyestrogens, particularly in Oriental women and a relative increase in 4-hydroxylation [41, 42], which means that the main metabolic pathways in these women unexpectedly seems to lead to biologically more active estrogens. However, it must be remembered that their plasma and urinary E1 and E2 levels were shown to be low [24] and the net biological estrogen effect may in any case be less. It has also been shown that the luteal phase E2 values are lower in young women following a low-fat diet for 2 months [49].

Women living in Africa consuming low-fat habitual diets [50] and Oriental migrants in Hawaii [24] have low plasma androgen levels compared with women on a Western diet. These observations are in agreement with the results obtained in postmenopausal omnivorous and vegetarian women and postmenopausal women with BC showing the lowest plasma A, T, %FT, %FE2 and DHEAS and highest SHBG (after correction for weight) in the vegetarian women, who had the lowest dietary fat/fiber ratio of the three groups [35, 43] The lower DHEAS in vegetarians is in agreement with recent results showing that plasma E1S levels are lower in women on a low-fat high-fiber diet compared to a typical Western diet [51] because the levels of these sulfates show a significant association [27 and unpublished results]. In correlation analysis a Western-type diet was found to be associated with the hormonal pattern observed in the postmenopausal women with BC, but this was obviously not entirely due to the diet [35].

It seems justifiable to conclude that a high protein and fat and low grain, complex carbohydrates and fiber intake leads to higher plasma levels of biologically active sex hormones and lower SHBG, with a clear tendency to lower 16α- and 16β-hydroxylation [42] and higher 2-hydroxylation of estrogens and higher urinary 2-hydroxy-E1/4-hydroxy-E1 ratio. The possible role of these alterations of hormone levels as etiological factors in hormone-dependent cancer will be discussed below. It should be mentioned that opposite results with regard to 16α-hydroxylation of estrogens and fat intake have been published [52, 53], and these results will be discussed in the section on BC.

LIGNANS, ISOFLAVONES, AND SEX HORMONE METABOLISM

Since the detection and identification of mammalian and later also of plant lignans and isoflavonic phytoestrogens in the human organism, many studies on their biological role in health and disease have been carried out. Several reviews [33, 54–56] on the topic have recently been published. These diphenolic compounds occurring in high amounts in the organism have numerous different biological activities of which most seem to make them candidates for a role as protective substances with regard to cancer and particularly hormone-dependent cancers [12, 21, 33, 34, 54, 56–64].

To date 15 lignans and isoflavonic phytoestrogens, all diphenolic in character, have been identified in human urine and some of them also in other biological materials [54, 56, 65, 66]. Of these 7 can now be measured by combined capillary gas chromatography–mass spectrometry utilizing the selective ion monitoring technique and isotope dilution mass spectrometry using deuterated internal standards [58, 67]. The lignans enterolactone (Enl), enterodiol (End) and matairesinol (Mat) and the isoflavonic phytoestrogens daidzein (Da), equol (Eq), O-desmethylangolensin (O-Dma) and genistein (Gen) have all weak estrogenic activity, but antiestrogenic activities have also been described [reviews in 54, 56]. Many plant lignans have been shown to have anticarcinogenic, antiviral, bactericidic and antifungal activities. In collaboration with Dr Larry Vickery (Irvine, California) it was shown that Enl and a theoretical

intermediate between Mat and Enl are moderate inhibitors of placental aromatase and compete with the natural substrate androstenedione for the enzyme. Enterolactone was also able to inhibit aromatase intracellulary in cell cultures suggesting that these compounds may function as natural aromatase inhibitors. Other experiments show that these diphenols are readily transferred from cell culture media into the cells and that they may inhibit cancer cell growth, because antiproliferative effects of synthetic and naturally occurring flavonoids on tumor cells of the human breast cancer cell line, ZR-75-1, were reported [59]. Furthermore, inhibitory effects of such compounds on mitogen-induced proliferation of human peripheral blood lymphocytes were demonstrated [60].

Genistein, one isoflavonic compound identified by us in human urine is a specific inhibitor of tyrosine-specific protein kinases [61-64]. Protein-tyrosine kinase activity is associated with cellular receptors for epidermal growth factor (EGF), insulin, insulin-like growth factor I (IGF-I), platelet-derived growth factor (PDGF) and mononuclear phagocyte growth factor (CSF-1), suggesting that the enzyme plays a role for cell proliferation and transformation. The enzyme has also been associated with oncogene products of the retroviral src gene family and is correlated with the ability of retrovirus to transform cells [literature in 61–64].

In collaborative studies with Dr Jim Clark and associates we have found that several plant and mammalian lignans and isoflavones compete with E2 for the rat uterine nuclear estrogen type II binding site (unpublished results). These sites seem to constitute a component of the genome which regulates estrogen-stimulated uterine growth [68, 69]. It was found that some flavonoids like luteolin, quercetin and pelargonin inhibit E2 binding to this receptor and in this way uterine cell growth. They also inhibited growth of MCF-7 cells in culture, and *in vivo* E2 stimulation of immature rat uterus [70]. The structure of these compounds are very similar to those of the isoflavones and in fact all are diphenols. The most effective with regard to type II site binding of the diphenolic compounds found and measured by us in human urine seem to be the isoflavones daidzein and equol, but also some lignans like matairesinol, isolariciresinol and enterolactone show competition (competition observed at concentrations from 10 to 100 nmol/l). Later an endogenous inhibitor of the nuclear type II binding site was identified as being methyl p-hydroxyphenyllactate [71], which can be a metabolite of both exogenous flavonoids and tyrosine. Because this compound cannot be found in cancer tissue it was postulated that uncontrolled growth and proliferation of malignant cells is directly related not only to the permanent stimulation of nuclear type II binding sites by estrogens or other compounds, but also to very low to nonmeasurable levels of the competitive inhibitor methyl p-hydroxyphenyllactate [71]. In our opinion it seems that probably many of these phenolic compounds may have a synergistic action as it is unlikely, because of close structural similarities, that only one of them inhibits cell growth. The compound found by Markarevich et al. [71] was isolated from fetal bovine serum, probably a very rich source of many flavonoids and phytoestrogens and their metabolites. The concentration of the new monophenolic compound in biological fluids and tissues in human subjects has to my knowledge not been measured. The possible growth-inhibiting and antiproliferative role of individual flavonoids and their metabolites with regard to hormone dependent cancer is a new interesting area of research that needs much further studies.

Of the isoflavones the strongest estrogens are Eq and Gen, but they are still very weak estrogens compared to E2 and E1. It is unlikely that all their other biological effects are related to their estrogenicity. Quantitative results indicate that lignans and isoflavonic phytoestrogens are normal constituents of human urine and are excreted in large amounts particularly by vegetarians (both lignans and phytoestrogens) [33, 34, 58], by subjects consuming large amounts of whole-grain products, vegetables and berries, which all are associated with high lignan excretion [33], and by the Japanese consuming traditional Japanese diet (mainly isoflavonic phytoestrogens, due to intake of soy products) [33, 72]. In omnivorous Finnish subjects the excretion of Gen, the specific inhibitor of protein tyrosine kinase, was found to be between

10 and 1,500 nmol/24 h (usually 1-4 times that of Da). When investigating a few Japanese subjects consuming a traditional diet the excretion was very high ranging from 1,250 to 15,500 nmol/24 h (!) (in collaboration with H. Honjo and coworkers), about 1.5 - 3 times higher than that of Da. As mentioned Da shows antiproliferative activity with regard to BC cells [59]. Particularly low excretion of these compounds has been observed in BC patients and in subjects consuming a low-fiber diet, especially a diet low in whole-grain products and beans [23, 24, 49, 64, and unpublished results]. Particularly low excretion has been observed in BC patients and in subjects consuming a low-fiber diet, particularly a diet low in whole-grain products [33, 34, 58, 73].

It has now been demonstrated that the mammalian lignans Enl and End are formed from precursors, such as the plant lignans matairesinol and secoisolariciresinol, which are consumed and then structurally modified by intestinal bacteria [56]. Eq and O-Dma are most likely formed by intestinal bacterial action from formononetin (For) and Da present in food stuffs like soy products [72, 74]. However, these compounds are also present in cow milk [75] formed from *e.g.* For in clover by intestinal bacteria in the gastrointestinal tract of the cow [55], and may therefore be consumed by human subjects as such. Because of the close association of lignan excretion with fiber intake [21, 33, 44] it is likely that the plant lignans are localized close to the outer fiber-containing layers of the grain containing phytin, polyphenols, enzyme inhibitors and other compounds usually regarded as antinutritional factors [76].

Recently, we suggested that the lignans and isoflavonic phytoestrogens, which all are diphenols, perhaps together with other similar compounds, stimulate SHBG synthesis in the liver and in this way reduce the biological effects of sex hormones [27, 33, 34]. An increase in SHBG results in lowering of %FT and %FE2 and reduction of both the albumin-bound and the free fraction of the sex hormones. This reduces the metabolic clearance rate (MCR) of the steroids and reduces in this way their biological activity.

In Finnish women total fiber intake, total fiber intake/kg body weight and grain fiber intake/kg body weight correlate positively and dietary fat/fiber ratio negatively with urinary excretion of total lignans and isoflavonic phytoestrogens [33, 34]. The excretion of the two diphenolic groups of compounds and also Enl alone in both pre- and postmenopausal Finnish women correlate positively with plasma SHBG and negatively with plasma %FE2 and %FT [33, 34 and unpublished results]. It is well known that oral estrogens, in contrast to parenterally administered ones, markedly stimulate SHBG synthesis [77, 78] and we therefore suggest that these positive associations between urinary lignan and phytoestrogen excretion and SHBG is due to stimulation of SHBG synthesis by these week estrogens entering the portal circulation in very high amounts. This also would explain the higher SHBG values seen in vegetarians [79] including such vegetarians whose diet does not contain low amounts of proteins [34]. High protein diet has been found to lower plasma SHBG [36, 37].

Furthermore urinary Enl excretion in these Finnish women correlates negatively with plasma DHEAS and luteinizing hormone (LH) (unpublished observations). The latter observation has to be evaluated in detail, but it is possible that the effect on sex hormone metabolism of these weakly estrogenic compounds may also be mediated via an effect on the hypothalamic–hypophyseal endocrine system. Plasma DHEAS is low in vegetarians and is negatively associated with the dietary intake of unsaturated fatty acids [35].

DIET, SEX HORMONES AND BREAST CANCER

In an extensive review about 10 years ago Dao concluded that studies of estrogen metabolism in BC has provided only controversial results and that they are inconclusive at best [80]. The results described above indicate clearly that studies on sex hormone metabolism in cancer cannot be carried out without careful dietary evaluation in the subjects studied. It is therefore not surprising that no consensus as to the association between sex hormone changes and BC has been reached, because very few studies include both detailed dietary records and hormonal investigations.

In his recent review Zumoff [81] includes nine hormone-related hypotheses in the discussion on hormones and BC, but none of them was discussed in relation to diet despite the huge amount of epidemiological data suggesting that a Western diet plays an essential role in increasing the BC risk in the Western world.

Because of the extensive literature I will discuss only a few of those hypotheses regarding the association of sex hormone alterations and BC, which seem to be related to diet.

The main change in diet when subjects from developing countries migrate to Western countries is an increase in animal fat and protein and a decrease in intake of complex carbohydrates, particularly whole grain products [10]. This change is identical to what has occurred in Scandinavia in the last 300 years and in fact has been going on in Finland since World War II with a simultaneous increase in the incidence of BC, CC and other Western diseases. I therefore like to discuss particularly the possible role in cancer development of complex carbohydrates like whole grain products and soy beans, cereal fiber and the role of lignans and isoflavonic phytoestrogense and their association with plasma SHBG and the % free sex hormones.

In two case-control [82, 83] and in an epidemiological study [84] it was shown that high fiber and high carbohydrate intake, respectively, decreased the risk of BC. In another case-control study particularly fiber from grains consumed during adolescence reduced the risk both in premenopausal and postmenopausal women [85]. These observations are in agreement with the results of our studies in postmenopausal women in Boston [35] and in premenopausal women in Helsinki [41] showing that the main and in fact only really significant difference between the diet of the BC patients and the omnivorous and vegetarian control women was a low intake of grain products and grain fiber. If the diets of the Boston and Finnish women studied by us are compared, the main difference is also in the grain and grain fiber intake, being much higher in the Helsinki women with a lower risk for BC than the Boston women. This dietary difference caused the mean fecal weights to be higher in the Finnish compared to the Boston women, despite similar mean total fiber intakes. The large fecal bulk affects the enterohepatic circulation of sex hormones, because there is *e.g.* a significant correlation between fecal weight and fecal estrogens. In both countries the fat/fiber ratio was the same in the omnivorous and BC women, but much lower in the vegetarian women, particularly in Boston, because the Finnish vegetarians consumed rather much fatty milk products. The postmenopausal Boston BC women had lower fat intake than the Finnish young vegetarians (!), the protein intake being similar. However, the fat to grain fiber ratio (g/g) was 16.4 in the old Boston BC women and only 10.2 in the young Finnish BC women and the corresponding values for the omnivores were 15.1 and 8.2, respectively. The Boston and Helsinki vegetarians had total fat/grain fiber ratios of 7.1 and 6.3, respectively. Very interesting are also the results of the protein/grain fiber (g/g) ratios in the six groups of women, The vegetarians, omnivores and BC patients in Boston and Helsinki had the following ratios: 7.2, 15.2, 18.1, and 5.4, 7.2 and 8.8, respectively. This shows that these ratios are very high in the omnivorous women and the BC patients in Boston, and also highest in the BC group in Helsinki compared with the other Finnish women mainly due to differences in grain fiber intake.

The fat intake in the BC women both in Boston and Helsinki was intermediate between that of the omnivores and vegetarians in respective city. This may be due to bias, particularly in Helsinki, because of much propaganda in this country about reducing fat intake in order to avoid cancer and other diseases. However, small differences in fat intake will not have any detectable effect on plasma or urinary sex hormone levels (for discussion see [12]), which may to some extent explain the results of a recent prospective study [86] in nurses that failed to show any correlation between high fat consumption and the subsequent development of BC (see also [87, 88]. However, in our opinion, after considering our above-mentioned results, it seems more appropriate to use the fat/total fiber or fat/grain fiber ratio to define the diet of risk groups and controls than to use % fat calories or total fat intake. However, recent prospective studies in our laboratory suggest that

particularly grain products containing all compounds of the grain may be protective and that so-called whole-meal products may be less satisfactory in this sense (see below). Also protein/carbohydrate or particularly protein/total fiber or protein/grain fiber ratio should perhaps be used to define the dietary groups. Using such ratios we have observed that the association of diet to sex hormone metabolism becomes much more obvious. We believe that this is related to the intestinal metabolism of hormones, lignans and isoflavones, which is dependent on the intestinal environment and closely related to our diet and perhaps better described or reflected by these ratios than by expressing the amounts of macronutritients as percentages of total calories or in relation to body weight.

Without doubt it is not fat alone which has the negative effects on overall sex hormone levels, but proteins, fiber and complex carbohydrates seem at least in Western societies to play even more essential roles. As an example of what this concept means is the increasing effect of a high fat and meat and low grain intake both in man and in experimental animals on intestinal β-glucuronidase (see literature in [21, 23]), which theoretically leads to an increase of the reabsorption of estrogens from the intestinal tract [25] and higher plasma estrogen levels [23, 24]. It should also be emphasized that the associations between fiber intake and the excretion of a number of urinary estrogens became statistically significant first when the fiber/kg body weight ratio was used instead of total fiber intake [26]. The fiber/kg ratio may better reflect the intestinal bacterial environment and fiber effects because a small subject has a smaller "internal" volume of the intestines compared to a tall subject.

The diet in Finnish rural areas where BC and CC incidence is low differs from the American one particularly with regard to its relatively high content of complex carbohydrates mainly from whole-grain products and starchy vegetables, the fat content being similar but deriving more from milk products than from meat [89, 90 and own observations]. A significant part of the Finnish milk product consumption consists of fermented milk products. Because of the differences in BC risk in USA and Finland we have postulated that this difference is at least partly due to the great difference in intake of whole-grain fiber-rich products like rye bread and perhaps some other fiber-rich nutritients such as berries. Particularly these foodstuffs increase the excretion of urinary lignans by the Finns and affect simultaneously also otherwise the intestinal milieau. This view was supported by the finding of very low urinary lignan excretion in the BC subjects living in Boston [57] and of lower excretion also in the young BC women in Helsinki [34, 73]. In both BC groups it was likely that the differences were due to low intake of whole-grain products. However, in Helsinki the differences between the omnivorious, vegetarian and BC groups were relatively small, because the grain intake was comparably high in all groups, which is typical for the original Finnish diet. It should be mentioned that the intake of wheat germ and bran do not at all cause increases in urinary lignan excretion in human subjects (own observations), and fiber-free wheat bread products have no or only very small influence on lignan excretion. Only grain products which have been made from milling of whole grain, without separating (and washing) the different components and mixing them again (R. Korpela and H. Adlercreutz, to be published) seem to significantly increase lignan excretion in Finnish women. This is because during modern milling of the grain, trying to eliminate so-called antinutritional factors [76], simultaneously also the diphenolic plant lignans seem to be at least partly eliminated. There are indications that also berries, fruits and various seeds [33, 56, 91] increase lignan excretion. Of some grain products, rye meal seems to result in the highest excretion of lignans in rats, followed in decreasing order by oat, barley and wheat meal [91]. The latter results are difficult to evaluate because no exact details were presented regarding the nature of the meal products consumed by the rats.

Based on an epidemiological study it was recently suggested that consumption of fermented milk products may protect against breast cancer [92]. In a case-control study consumption of fat from milk, cheese and yogurt during adolescence reduced the BC risk both in premenopausal and postmenopausal women [85]. One mechanism by which fermented milk may influence hormone metabolism

is by reduction of the β-glucuronidase-producing bacteria of the intestinal content [93, 94], which theoretically should reduce the enterohepatic circulation of estrogens and increase the fecal route of elimination. The conjugated estrogens excreted in the bile must be deconjugated before the estrogen moiety can be reabsorbed. Milk products have also been found to contain animal lignans and isoflavonic phytoestrogens (75) and even if the concentrations are rather low they add to those produced by the intestinal bacteria from plant precursors.

Our hypothesis has been that high intake of whole-grain products (preferably in combination with reduced fat and moderate protein intake) reduces BC (and CC) risk because such a diet increases fecal bulk and reduces intestinal β-glucuronidase activity and steroid and bile acid enterohepatic circulation and results in increased mammalian lignan production [12, 21]. Later on we also included the isoflavonic phytoestrogens into the original theory [33, 54]. This was due to the finding of very high excretion of isoflavonic phytoestrogens in urine of Japanese men and women consuming a traditional diet [33, 72]. The lignan excretion in the Japanese subjects was low, even lower than we found in the postmenopausal BC patients in Boston. The isoflavones resemble lignans with regard to structure (all are diphenolic). In most correlation studies they show parallel behaviour. In the Finnish women the significances of the positive correlation between the excretion of lignans and isoflavonic phytoestrogens in urine, and plasma SHBG, and the negative correlations with %FE2 and %FT are stronger than the separate correlations for each group of compounds [33]. Recently, our hypothesis with regard to the protective role of these compounds for BC got strong support from studies showing that powdered soy bean chips, both before and after denaturation of protease inhibitors, decrease mammary tumor formation in a rat breast cancer model [95]. Furthermore Gen, found by us in human, chimpanzee and cow urine, may be anticarcinogenic due to its inhibitory effect on protein tyrosine kinase [61–64] and other flavonoids are antiproliferative with regard to BC cells [59]. The postmenopausal BC patients in Boston had the lowest plasma SHBG and highest %FT and %FE2 [35] and the lowest Enl and Eq excretion [57]. The Finnish premenopausal BC subjects had lower SHBG, higher %FT and %FE2 and lower excretion of lignans and isoflavonic phytoestrogens compared to the vegetarians [34]. In many studies low SHBG has been associated with BC (see literature in [35, 96]).

Because of the large differences in grain fiber intake and urinary lignan excretion between postmenopausal women living in Helsinki and Boston we have in preliminary calculations combined the materials of postmenopausal women and found the same highly significant positive correlation between grain fiber intake or Enl excretion and plasma SHBG and negative correlations with plasma %FE2 and FT (unpublished observations) as we found for the young Finnish women [33, 34].

The theory based on the observation that high fat intake increases 16α- and decreases 2-hydroxylation of estrogens leading to biologically more active estrogens also needs some discussion. According to this theory a low rate of 2-hydroxylation and high rate of 16α-hydroxylation leads to a greater risk for BC and endometrial cancer [52, 53, 97–99] because 2-hydroxylated estrogens are biologically less active than 16α-hydroxylated ones. Several earlier studies as well as our own seem to speak against this hypothesis because all low-risk groups, compared to high-risk groups, have relatively more urinary 16α-hydroxylated estrogens, particularly if also the fecal estrogens are included. Women living in low-risk countries consume most of their calories in the form of complex carbohydrates and have lower fat and protein intake, which should lead to low 2-hydroxylation of estrogens [38, 39]. This we could observe in the young premenopausal Finnish women [40, 41] and in the previously investigated Oriental women [23, 42]. The characteristics of the sex hormone pattern in these low-risk Oriental women on a low-fat diet are low plasma levels of E1, E2, A and T and low excretion of E1, E2 and 2-hydroxylated estrogens and relatively high amounts of both 16α- and 16β-hydroxylated estrogens [23, 42] We could also not see any increase in 16α-hydroxylated estrogen metabolites in urine of Finnish premenopuasal women with

BC. In fact slightly higher mean values were seen in the vegetarians, but the differences were not significant [40, 41].

Recently we completed the second part of the Finlandia study dealing with groups of postmenopausal women and found results apparently more in line with those suggesting that high 16α-hydroxylation is a risk factor. A statistically significant (logarithmic) negative correlation between plasma SHBG and urinary 16α-hydroxyestrone ($R = 0.59$, $p < 0.001$) and estriol ($R = 0.49$; $p < 0.01$) was found with the highest values of estriol and lowest SHBG values in the BC and omnivorous women and higher SHBG and lower urinary 16α-hydroxylated estrogens in the vegetarians. In the same material there was a significant positive correlation between urinary total diphenol excretion and plasma SHBG ($R = 0.64$; $p < 0.001$). From our results it appears that the tendency to lower values of 16α-hydroxylated estrogens in urine of the vegetarian and higher in the omnivoric and BC women is probably due to different degrees of fecal elimination of these estrogens as a result of differences in fiber intake and not to increased 16α-hydroxylation of estrogens in BC. However, the evaluation of this very large study is still in progress and the definite results have to await the extensive statistical treatment needed. In these postmenopausal women we found no correlation between plasma SHBG and urinary catecholestrogens but a highly significant positive association between the logarithms of plasma E1S and urinary excretion of 2-hydroxy-E1 ($R = 0.84$; $p < 0.001$). The BC women tended to have both higher plasma E1S and urinary 2-hydroxy-E1, which supports our theory that high E1 and E1S and urinary catecholestrogens may be risk factors of BC. It may be mentioned that high E1S has also been found in EC [100].

With regard to 2-hydroxylated estrogens there is evidence speaking for a role of these steroids and catecholestrogens formed from stilbestrol in hormonal carcinogenesis via microsome-mediated redox cycling and formation of quinones and free radicals [101]. The quinoid structures are prerequisites for the genotoxic effect [102] because they are capable of covalent binding to proteins [103, 104]. The development of renal tumors in Syrian hamsters after estrogen treatment has been postulated to occur via a free radical mechanism [105]. Hydroxylated flavonoids have antagonistic effects on the mutagenic and/or tumorigenic activity of epoxide metabolites of polycyclic aromatic hydrocarbons [106]. Because of similar structure the isoflavones and lignans should also be investigated in this respect.

DIET, HORMONES, LIGNANS AND ISOFLAVONES, AND OTHER WESTERN DISEASES

It is not possible in this connection to discuss at any length the relationships between diet and other Western diseases. Some very large reviews on nutrition and its relationship to cancer have been published [107, 108]. However, I would like to discuss shortly some new results indicating that the above discussion may have some important implications also for other diseases than BC and that obvious hormonal and biochemical connections exist between BC and other Western diseases.

Endometrial cancer

What has been said about diet and estrogen metabolism and BC holds as well for EC, a disease even more clearly estrogen-dependent than breast cancer. An increase in bioavailable estradiol due to lowering of SHBG and increase in reabsorption of biliary estrogens as a result of a Western diet would also promote the growth of endometrial cancer. This cancer type has in addition been found to be associated with other diseases common in the Western world, like hypertension and diabetes. Hypertension has in fact recently been found to be a risk factor also of BC [109].

Prostate cancer

Furthermore, it is known that a low-fat and/or high-fiber diet affects sex hormone metabolism also in men [28–30] by decreasing T and FT. A high level of biologically active androgens probably accelerates the development of PC in the Western world and recently a prospective study in fact seems to indicate that elevated T levels are

associated with increased risk of PC [110]. In epidemiological studies fat and meat show a positive and cereals a negative association with PC mortality [3]. In Japan and some other Asian countries, despite the same incidence of latent small or non-infiltrative prostatic carcinomas, the mortality is low [111–113]. This could at least partly be explained by a diet-related lowering of biologically active androgens as seems to occur in Asian women [24] and in the above-mentioned experimental studies [28–30]. Rotkin (cited from [114]) suggested that the men at risk of developing PC had a "strong overbalance of androgenic components" and observed that fewer patients with prostatic cancer developed gynecomastia and obesity early in life compared to controls. However, also recent observations indicate a possible protective effect of endogenous estrogens [115, 116] and this would suggest that the high levels of isoflavonic phytoestrogens in the traditional diet of Japanese men [33, 68] may also represent a protective factor [33, 54] inhibiting the growth of already existing small cancers [theory originally proposed in 117]. However, other than estrogenic effects of these substances may be more important.

The above-mentioned theory gains support from the recent observations of decreased risk of prostate cancer in Adventist men showing high consumption of beans, lentils, peas and some dried fruits (dietary sources of flavonoids) [118] and in men of Japanese ancestry in Hawaii consuming much rice (mainly starch, which may have some fiber-like effects in the gut) and tofu [119], a soy bean product. Our own results in Japanese men and women [some results in 72] show a strong positive association between the intake of various soy products and urinary excretion of equol and daidzein, and also a positive correlation with lignan excretion, particularly enterodiol, despite the fact that lignan excretion was low in the Japanese subjects investigated. It was in fact suggested [112], that if new small latent carcinomas are being formed at a constant rate they may either disappear or may enlarge and develop into larger carcinomas in different numbers or at different speeds in different geographical areas. It is suggested that in certain populations dietary factors affect androgen metabolism and biological activity as described above and/or that dietary isoflavones and other phytoestrogens directly influence cancer cell growth slowing the speed of development of these small latent carcinomas. The possible effect of soybean diets on PC may be a parallell to the observation of the inhibitory effect of this diet on breast tumor incidence in experimental animals [95, 120].

Coronary heart disease

Low SHBG has been found to be a risk factor of CHD mortality in a female population during a 12-year follow-up period [121] and is probably a risk factor also in men [122]. In addition, low plasma 5α-DHT seems to be a risk factor of CHD in men [122, 123]. As mentioned previously a high dietary protein/carbohydrate ratio not only suppresses plasma levels of SHBG, but simultaneously inhibits liver 5α-reductase [36–39]. Furthermore, we found significantly higher SHBG and HDL-cholesterol and almost significantly higher 5α-DHT ($p < 0.07$) in joggers compared to the subjects with CHD and a positive association between SHBG and HDL-cholesterol and maximal oxygen uptake in both joggers and healthy men [122]. Plasma SHBG and 5α-dihydrotestosterone concentration correlates positively with HDL-cholesterol and apoliprotein A-I both in healthy middle-aged men and in men with CHD [122, 123]. It is also known that thyroid hormones and estrogens stimulate SHBG synthesis, increases liver 5α-reductase and plasma HDL-cholesterol and apolipoprotein A-I [124, 125]. In population studies HDL-cholesterol and apolipoprotein A-I are inversely related to CHD [126, 127]. Compounds increasing the 5α-/5β-reductase activity ratio in rat liver microsomes lower serum cholesterol and reduces the incidence and severity of atherosclerotic lesions in aortas of cholesterol-fed rabbits [128]. Whether the higher plasma SHBG and 5α-DHT in our physically fit men compared to the subjects with CHD is due to diet or to physical exercise itself cannot be judged at present. The protein/carbohydrate ratio of the diet may be lower in hard-training joggers, which could explain the high SHBG and 5α-DHT levels. This is because aerobic training usually leads to increased proportion of carbohydrates in the diet.

In Finnish men this may mean increased consumption of whole-grain rye products, because about 40 % of the cereals consumed in Finland are rye products [see *e.g.* 129] and rye bread is usually a whole-grain product in this country.

As already mentioned consumption of whole-grain rye bread has recently been found by us to considerably increase animal lignan excretion in urine (R. Korpela, H. Adlercreutz, to be published), and it also seems to stimulate SHBG synthesis (almost statistically significant increase after 2 weeks; $p < 0.07$) as suggested previously [33]. Furthermore, it is of interest that isoflavones, excreted in high amounts in urine in populations having a low CHD risk, like the Japanese men, have hypocholesterolemic effects in rats [130] and that treatment with a soybean-protein diet has remarkable hypocholesterolemic effects in human subjects with type-II hyperliproteinemia [131]. Soybean protein products contain isoflavonic phytoestrogens, but whether the effect observed is due to these compounds or to the plant protein itself, as suggested by the authors, is uncertain. It is interesting to note that it has been suggested that the hypocholesterolemic effects of isoflavones is probably independent of the estrogenic effects [130]. Furthermore, it has been shown that the hypocholesterolemic effect of soy products in human subjects is not due to the content of soybean fiber [132]. It is concluded that very similar associations between diet, SHBG, lignans and isoflavones, as found for BC, seem to exist also with regard to CHD.

Colon cancer

In epidemiological studies a parallelism has been observed between BC and CC [133], but there are also some discrepancies suggesting different etiology [review 86]. However, for none of the Western diseases the etiology is likely to be monofactorial and looking only for the associations with macronutritients may easily lead us to wrong conclusions. There are also some parallelisms between CC and PC [3], and diet, in the majority of the opinions [107,108], seems to be the most important environmental factor in the development also of CC.

CC has also been found to be related to reproductive and hormonal factors [review in 130] and it has been found that increasing parity decreases risk and late age at first live birth increases risk [135, 136] as found also for BC. Women with cancers of the breast and other reproductive sites have an excess of primary colorectal cancer and pregnancy protects against DMH-induced colon cancer in experimental animals [review in 136]. Many colon tumors contain sex hormone receptors [137-140], and they may play a role in the pathogenesis of the disease [141].

The observed discrepancies in parallelism between CC and BC incidence and mortality development in Japan [86] may be due in addition to changed consumption of macronutritients to some micronutritients like plant lignans and isoflavones having a large spectrum of biological activities like anticarcinogenic, antiproliferative, antihormonal or hormonal and antiviral effects, which may play a role also locally in the intestine [21, 142]. The local effects in the intestine may be independent of the formation of the hormonally active substances which seem to alter liver and peripheral sex hormone metabolism. Another factor which may play a role for the discrepancies in parallelism between CC and BC is that a change in the fat content of the diet e.g. in Japan may not parallel a change in the use of soy products, because the soy sauce is mainly used for its content of sodium chloride and other soy products may still be used independently of an increase in fat intake. When leaving the habit to consume a low-fat diet the Japanese seem to still consume rice and they do not get any additional (cereal) fiber needed to compensate for the higher fat intake, because whole-grain bread seems to be almost unknown in Japan. This in our opinion could perhaps explain that the CC incidence in Japan increases more rapidly than the BC incidence [86] because of the absence of cereal fiber but continuous consumption of soybean products and rice.

Furthermore, an increase from 10 to 25 % of the fat calories as has occurred in Japan between 1955 and 1975 [86] may not alter the hormonal pattern as much as the difference we find for urinary and plasma sex hormones when the fat calory intake is

about 20 % compared with that found when it is about 38 % [24, 42]. In our own studies in a rural village outside Kyoto [72] women and men still consume only 20 and 17 % fat calories, respectively.

In most epidemiological studies a relation between fat intake and CC has been observed, but in only few studies an association has been found between CC risk and high protein intake or high energy consumption [143, 144] both leading to low SHBG, despite the fact that fat and protein consumption generally increase in parallel. In one study a high meat/vegetable consumption ratio predisposed for CC [145], a diet, which probably also would affect sex hormone pattern [35].

However, as for BC, a negative association between CC and intake of cereals or nonstarch polysaccharide fiber has been observed in most (but not all) epidemiological studies [review in 146, 147], the case-control studies being less convincing [see 147]. To my knowledge no prospective studies on effect of grain fiber or whole-grain products on CC incidence have been published. Recent studies suggest that the fat/fiber ratio is important also in the pathogenesis of CC because a negative association between CC and dietary fiber was found only in men with low fat consumption [148]. Epidemiological studies in Finland and Denmark point to a protective role of cereal fiber [89, 90, 129, 149], but also other factors like high consumption of fermented milk lowering colonic pH [150, 151] and supplying calcium [152, 153] [review in 154] are most likely partly responsible for the favourable CC incidence in rural Finland. Thus fermented milk may play a role for both BC (lowering effect on intestinal ß-glucuronidase) and CC risk [94, 154, 155]. As indicated above the dietary fat/fiber ratio seems to determine the degree of the enterohepatic circulation of hormonal steroids and may in this way alter the risk of hormone-dependent cancers. In experimental colon carcinogenesis this ratio determines the tumor prevalence and dietary fiber content determines the bile acid concentration and protects against the deleterious effects of fat [156, 157].

Because of the relatively high consumption of whole-grain rye bread in Finland we have been interested in studying whether different cereal products may have different effects on the CC risk factors. From these studies we have now obtained more support for the theory [21] that certain fiber-rich grain products, supplying precursors for mammalian lignan formation perhaps protecting against BC and locally having a favourable influence on intestinal bacterial composition and metabolism and mucosal cell environment, may be protective with respect to CC also by another mechanism. This is because rye bread seem to favourably influence intestinal bile acid metabolism. In a recent experiments we observed that by changing the bread consumption from a wheat fiber-free bread or from a whole-meal fiber-rich (fiber > 9 %) wheat bread to a whole grain rye bread (fiber > 8 %), significant alterations of the biochemical risk factors of CC could be obtained (see below), suggesting that the relatively small dietary change may have positively affected intestinal metabolism. The rye bread made from whole grains, not purified during milling, compared to both the fiber-free and a fiber-rich wheat bread (produced after modern milling of the grain eliminating some fractions, but containing essentially all components) increased considerably the urinary lignan excretion (R. Korpela & H. Adlercreutz, to be published). Compared to the control period no change (whole-meal) or a decrease (wheat, fiber free) was observed for the other breads. As mentioned above we have shown that lignan excretion is low in women with BC [21, 34, 57], most likely due to low intake of whole-grain bread. Furthermore, it has been shown that autohydrolyzed lignin, which is a polymer with similar basic structure as the diphenolic lignans, protects against experimental colon adenocarcinoma in rats [158]. Lignin is also known to bind deoxycholic acid very well compared to other types of fiber [159]. The effect of rye bread (200 – 300 g per day, no other cereal products consumed) on intestinal bile acid metabolism was remarkable because it considerable decreased the total free bile acid, and total and free secondary bile acid concentrations and the ratio of secondary to primary bile acids in feces (J. T. Korpela, H. Adlercreutz & R. Korpela, to be published) leaving, however, the LCA/DCA ratio unchanged. This ratio increased with consumption of the fiber-free

wheat bread. The reason for the decrease in free bile acids was a huge increase in the concentration of saponifiable (esterified) bile acids to a mean of about 46 % of total bile acids. These esters have been found to form a high proportion of the bile acids in feces in vegetarians (up to 80 %) but occur in very low amounts in CC patients (mean about 10 % of total bile acids) [160]. According to our theory the saponifiable (esterified) bile acids may not be cocarcinogenic or comutagenic as found for the aminoconjugates of these acids [16]. The reason for this may be that they are nonpolar and therefore less water-soluble which may be advantageous [152]. With the other types of bread practically no change of bile acid pattern occurred, or if any, it was in the opposite direction, particularly with respect to the fiber-free wheat bread. This is in agreement with a previous study showing no change in fecal bile acid excretion after consumption of a wholemeal bread compared to "white bread" [161]. In this connection it is of interest to note that during wartime the milling of flour resulted in much higher fiber, and possibly lignan precursor contents, which seems to have resulted in a modest decrease in colon cancer mortality [162]. These results would imply that by a simple change of the bread consumption to a daily intake of 200 – 300 g of whole-grain rye bread (or some other grain?), containing all the components of the cereal, the risk for both BC and CC could at least theoretically be reduced. Interestingly recent associations have been found both between BC [163] and CHD [164], and adenomatous polypes in colon, which are regarded as the first stage of some CC tumors.

Our results with respect to fecal bile acid metabolism are not in disagreement with the original theory of Hill *et al.* [13], but extend the theory to include the degree of "esterification" (the saponifiable bile acids have not yet been characterized). It is still most likely that the concentration of free secondary bile acids is an important factor determining the CC risk [15–20, 165, 166].

CONCLUSIONS

In conclusion, it seems that a Western diet with high fat and protein intake and low intake of fiber, complex carbohydrates and whole-grain products is associated with high plasma sex hormone levels and low SHBG, 5α-DHT, high %FT and %FE2, high urinary and low fecal excretion of estrogens, high urinary catecholestrogens excretion and 2-hydroxy-E1/4-hydroxy-E1 ratio, and low urinary excretion of lignans and isoflavonic phytoestrogens. These compounds apparently are protective with regard to cancer by many different mechanism. With respect to plasma hormones (except 5α-DHT), urinary lignans and equol we found this pattern in the postmenopausal BC women in Boston. Furthermore such a diet leads to unfavourable plasma lipid levels and intestinal bile acid metabolism most likely increasing the risk for both CHD and CC. In the study in Finland, where the BC and CC incidences are much lower than in USA, the hormonal pattern in the young BC patients was very similar to that of the control omnivorous and vegetarian women (33, 34), probably because of the relatively high intake of grain products [41] in all groups studied, but mean grain intake was still lowest in the BC group. The situation may be different in premenopausal compared to postmenopausal women, but still nothing speaks against the theory that diet is an important BC risk factor. This seems to be the fact particularly in the postmenopausal women, but probably and perhaps to a lesser degree, also in young women. All dietary components seem to have their specific role(s) in influencing sex hormone metabolism as described above and in this way a wrong diet may influence the development of BC and other sex hormone-dependent cancers in the promotional stage of the disease. More work is still needed, but already now it seems that the above-mentioned studies showing very distinct associations between diet and sex hormones and SHBG and diet and fecal bile acid pattern fit rather well with the view of the epidemiologists, that Western diet is the main factor causing the high incidence of hormone dependent cancers and CC in the Western world. Furthermore, many significant biochemical and hormonal connections between BC and other Western diseases, like CHD, exist, indicating that the same type of diet partly by the same mechanisms may be responsible for several of these diseases.

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DIETARY PHYTOESTROGENS AND CANCER: *IN VITRO* AND *IN VIVO* STUDIES

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Summary—Thirty postmenopausal women (11 omnivores, 10 vegetarians and 9 apparently healthy women with surgically removed breast cancer) were investigated with regard to the association of their urinary excretion of estrogens, lignans and isoflavonoids (all diphenols) with plasma sex hormone binding globulin (SHBG). A statistically significant positive correlation between urinary total diphenol excretion and plasma SHBG was found which remained statistically significant after elimination of the confounding effect of body mass determined by body mass index (BMI). Furthermore we found a statistically significant negative correlation between plasma SHBG and urinary excretion of 16α-hydroxyestrone and estriol which also remained significant after eliminating the effect of BMI. Furthermore we observed that enterolactone (Enl) stimulates the synthesis of SHBG by HepG2 liver cancer cells in culture acting synergistically with estradiol and at physiological concentrations. Enl was rapidly conjugated by the liver cells, mainly to its monosulfate. Several lignans and the isoflavonoids daidzein and equol were found to compete with estradiol for binding to the rat uterine type II estrogen binding site (the s.c. bioflavonoid receptor). It is suggested that lignans and isoflavonoids may affect uptake and metabolism of sex hormones by participating in the regulation of plasma SHBG levels and in this way influence their biological activity and that they may inhibit cancer cell growth like some flavonoids by competing with estradiol for the type II estrogen binding sites.

INTRODUCTION

Weakly estrogenic diphenolic compounds, belonging to the classes of lignans (Ligs) and isoflavonoids (Ifls), are excreted in large amounts in human (and animal) urine. Subjects consuming whole-grain products, seeds, fruits and berries (contains mammalian lignan precursors) and soy products (contains isoflavonoids, and lignan precursors) [1–6] have high excretion of these compounds. Up to now about 15 structurally different compounds were isolated and identified by combined gas chromatography–mass spectrometry (GC/MS) [structures and literature in 4, 6, 7]. Intestinal bacteria play an important role in the transformation of the plant precursors [2, 7, 8].

Lignan excretion in women is usually high in areas with low risk for breast cancer (BC) like North Karelia in Finland [4], and in vegetarians [4, 5, 9, 10] and low in women living in high-risk areas like Boston, U.S.A. [4, 5, 10]. In old women with BC in Boston the excretion was very low [10] and it was also relatively low in Finnish young women with BC [9]. On the other hand we also found low excretion of Ligs in Japanese women consuming traditional Japanese diet and having low BC risk. However these subjects excreted very high amounts of Ifls, particularly genistein (Gen) and daidzein (Daid) [11]. There is already evidence suggesting that both Ligs and Ifls are protective with regard to BC [12–17] and that Ifls may be protective with regard to prostate cancer (PC) [16, 18].

In the present study we continue to explore the link between the Ligs and Ifls, and hormone-dependent cancer and the possible mechanisms by which the cancer-protective effect of these compounds is exerted. The results obtained strongly suggest that these compounds have cancer-protective properties.

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MATERIALS AND METHODS

Subjects and their diet

In this connection only some preliminary *in vivo* results with regard to plasma sex hormone binding globulin (SHBG), urinary estrogens, Ligs and Ifls for the last part of the "Finlandia" study involving postmenopausal women will be described. The groups studied were 11 omnivorous and 10 vegetarian women and 9 apparently healthy women with breast cancer (BC) treated with surgical removal of the breast (Stage I and II). Simultaneously with the collection of urine and blood samples, very careful dietary records during 5 days were obtained, once in winter and once in the summer time. The dietary differences were surprisingly small. Preliminary calculations showed that the vegetarians had higher intake of total fiber (22.7 g/day, geometric means) than the omnivores (16.6 g/day) and the BC patients (16.0 g/day) but this was statistically significant only when compared with the BC group ($P < 0.04$). No significant differences in dietary intake between the omnivorous and BC groups could be observed. Cholesterol intake was significantly lower in the vegetarians (omnivores vs vegetarians $P < 0.02$; BC vs vegetarians $P < 0.002$). Furthermore we found a statistically significantly higher intake of vegetable fiber in the vegetarians compared to the BC group (vegetarians 4.5 g/day and BC 2.3 g/day, $P < 0.03$). Complete dietary data will be published elsewhere.

Collection of blood and urine samples

The women collécted 72-h urine samples and three different blood samples were drawn between 8 and 9 a.m. into heparinized tubes on the same consecutive days. The plasma was pooled and the samples were stored with 0.1% ascorbic acid and 0.1% sodium azide at −20°C until analyzed. In the present study the mean values for one winter and one summer collection period were used (6 plasma and 2 × 72-h urine samples for each subject).

Reference standards and deuterium-labelled compounds

The Ligs Enl, End, matairesinol (Mat), and the Ifls, Daid, Equol and *O*-desmethyl-angolensin (*O*-Dma) were synthesized and the preparation of the deuterium-labelled standards was carried out as described previously [lit. in 19]. The isoflavonoid Gen was a generous gift from Professor K. Kallela.

Cell cultures

Prior to the growth experiments the cells (HepG2 liver cancer and MCF-7 breast cancer cells, American Type Culture Collection, Rockville, Md, U.S.A.) were maintained in Dulbecco's modified Eagle's medium (DMEM) without phenol red supplemented with 10 mM of L-glutamine, 100 IU/ml of penicillin, 100 IU/ml of streptomycin, 1% (v/v) NEAA, and 15 mM Hepes (Boehringer Mannheim, Fed. Rep. Germany) with 10% fetal calf serum (FCS). Before the experiment the cells were detached by removing the medium and washing with ice-cold Ca- and Mg-free phosphate buffered saline (PBS) (Orion Diagnostics, Espoo, Finland) and trypsinization (trypsin 0.05%, EDTA 0.02%). 100,000 to 400,000 cells, depending on the size of the plastic petri dishes, were plated in the same medium but now with 5% FCS for two days. The medium was removed, cells washed with PBS, and fresh medium with 5% twice DCC-treated FCS added and incubated for a further 3 days. The preparation of the DCC-treated FCS was carried out as described [15].

After the final washing of the cells twice with ice-cold PBS and added fresh medium with 5% DCC-treated FCS, the cells received effectors in ethanol solution to a final concentration of not more than 0.1% ethanol. The cells were maintained at 37°C in a 100% humid atmosphere of 92% air and 8% carbon dioxide as a monolayer culture in Falcon's plastic petri dishes (9 cm dia.) or in dishes with six 2.5 cm wells. The effectors were added once per day and the medium changed every fifth day. Duration of experiments was 8–10 days. Cells were counted both manually in a Bürker chamber and using the Coulter counter industrial cell counter (Coulter Electronics Ltd, Luton, Beds., England). DNA was measured by fluorometry with a slight modification of the original procedure [20] using the Transcon 102 FN fluoronephelometer (Elomit Oy, Helsinki, Finland) and the results were expressed in pmol/mg DNA.

Cell cultures in metabolite studies and determination of enterolactone conjugates

In the metabolite studies with HepG2 cells, the cells, after the initial treatment described above, were first grown for four days as described and every morning Enl was added to a final concentration of 1 μM. After four days the medium was removed and the cells washed with

ice-cold PBS and fresh medium added. Thereafter the procedure was continued for another 4 days and 24 h after the last addition of Enl extraction was carried out as described [15]. The fractionation of Enl conjugates and their determination was carried out as previously described [21] with slight modifications [15]. To the final fractions 211.2 ng $^2H_6$-labelled Enl was added in 50 μl of ethanol, the solvent evaporated to dryness and the samples silylated. After trimethylsilyl ether derivative formation the solvent was evaporated to dryness, the residue was dissolved in a suitable amount of n-hexane and the quantitation carried out by GC/MS in the selected ion monitoring (SIM) mode as described [22].

In studies on the time course of Enl conjugation, 1 million HepG2 cells were plated and Enl added to a final concentration of 2 μM. Samples were taken at various time intervals up to 74 h. Medium was extracted with ethyl ether and the conjugates hydrolyzed with *Helix pomatia* extract as described [22] and the liberated aglycone extracted with ether and assayed by GC/MS.

Determination of SHBG in the medium

SHBG assays in the medium were carried out with a highly sensitive time-resolved fluoroimmunoassay (TR-FIA) using reagents provided by Farmos Ltd (Turku, Finland).

Studies of the binding of diphenols to the nuclear type II estrogen binding site

Adult ovariectomized rats were implanted with 20 μg of estradiol (E2) and 96 h after treatment uterine nuclear fractions were prepared. The various lignans and isoflavonoids were dissolved in Tris–EDTA buffer containing 20% ethanol and their ability to inhibit the binding of [^{3}H]estradiol (40 nM) to nuclear type II sites were assessed [23, 24].

Assays of estrogens, lignans and isoflavonoids in urine and SHBG in plasma

Ligs and Ifls were determined in urine by an isotope dilution gas chromatographic–mass spectrometric method recently described [19] combining the method with the estrogen profile method also described previously [22]. This allows simultaneous assay of 20 compounds. In the present study Mat and Gen were not assayed because the method did not include these two compounds at the time of analysis. Thus we determined 13 estrogens and the Ligs Enl and End, and the Ifls Daid, Equol, and *O*-Dma. SHBG in plasma was determined by the RIA kit provided by Farmos Ltd.

Statistical methods

The mean values presented are geometric means. In the statistical analyses the mean values for the winter and summer collection periods were used and when necessary logarithmic transformation was made because of skewness of the distribution of the results. The degree of univariate associations between two variables was estimated as Pearsons correlation coefficients (r). Partial correlations were calculated to eliminate the effect of body mass index (BMI) on the results. Correlation coefficients and partial correlation coefficients were calculated using the StatView II programme for Macintosh II (Abacus Concepts, Inc. Berkely, CA, U.S.A.).

RESULTS

SHBG, diphenols and estrogen 16α-hydroxylation

In the three groups of postmenopausal women the plasma SHBG values were statistically significantly highest in the vegetarians (70.3 nmol/l) ($P < 0.0002$) compared to the omnivores (31.1 nmol/l) and BC patients (34.8 nmol/l). The vegetarians had significantly higher urinary excretion of Enl, total Ligs, total Ifls, and total diphenols ($P < 0.05$–$P < 0.007$) compared to the two other groups (details to be published elsewhere).

We found a statistically significant positive correlation between urinary excretion of *O*-Dma, Enl, total lignans and total diphenols and plasma SHBG. However, this correlation was partially dependent on the fact that the vegetarians had significantly lower body mass (BMI = omnivores 26.1, vegetarians 22.4, BC 26.2). BMI showed a negative correlation with SHBG ($r = -0.580$; $P < 0.001$) in these subjects. After elimination of the confounding effect of BMI we still found a statistically significant positive association between the urinary excretion of *O*-Dma ($r = 0.421$), Enl ($r = 0.391$), total Ligs ($r = 0.382$) and total diphenols ($r = 0.400$) and plasma SHBG ($P < 0.05$ for all).

When studying the association between plasma SHBG and the excretion of individual urinary estrogens we found that there was no association between SHBG and urinary catecholestrogens. However, we found statistically

significant negative correlations between SHBG and urinary 16α-hydroxyestrone ($r = -0.448$; $P < 0.05$) and estriol ($r = -0.572$; $P < 0.001$). Partial correlation coefficients eliminating the linear effect of BMI on the results showed that these significant associations remained but were weaker ($r = -0.390$ and $r = -0.409$ for 16α-hydroxyestrone and estriol, respectively, both $P < 0.05$).

Stimulation of SHBG synthesis by enterolactone in HepG2 liver cell cultures

Concentrations of Enl between 0.5 and 10 μM stimulated SHBG synthesis by HepG2 human liver cancer cells in culture (Fig. 1). The maximal effect was found with 5 μM concentration and a toxic effect could be observed with concentrations above 10 μM (Fig. 1). 200 nM concentration of estradiol (E2) was needed to obtain a similar stimulation of SHBG synthesis as 2 μM of Enl. When E2 (200 nM) and Enl (5 or 10 μM) were combined they had additive effects on the synthesis (Fig. 1).

Metabolism of enterolactone by HepG2 liver cells

The conjugation of Enl by HepG2 cells was very rapid and within 10 h more than 95% was conjugated (Fig. 2). The relative concentrations of the different conjugates of Enl identified in the medium are shown in Table 1. The main conjugate was the monosulfate (EnlS) amounting to about 78% of the total.

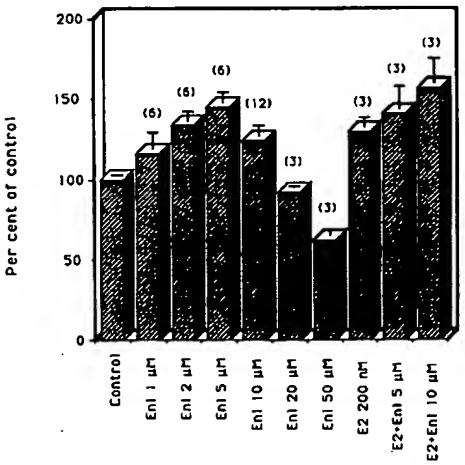

Fig. 1. Enterolactone stimulation of sex hormone binding globulin (SHBG) synthesis by HepG2 cells in culture in the absence and presence of estradiol. Number of experiments indicated on the top of the bars.

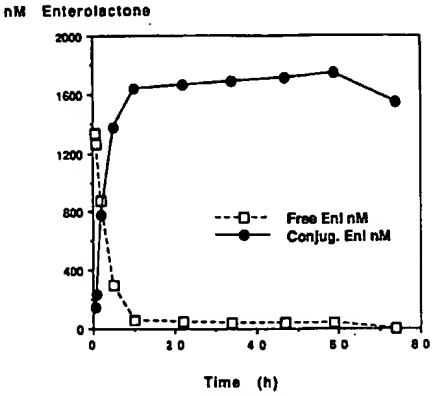

Fig. 2. Conjugation of enterolactone (Enl) (2 μM) added to cultures of HepG2 cells.

Binding of diphenols ot the nuclear type II binding site (bioflavonoid receptor)

Figure 3 (top) shows the binding of the two main mammalian lignans Enl and End to the nuclear estrogen type II binding site. In addition the binding of two plant lignans, matairesinol, which is the precursor of Enl [2, 7] and of isolariciresinol is shown. In the lower part of the figure it can be seen that daidzein and equol show significant binding but their precursor formononetin does not bind to the bioflavonoid receptor.

DISCUSSION

It has been proposed that a low rate of 2-hydroxylation and high rate of 16α-hydroxylation leads to a greater risk for BC and endometrial cancer. BC patients, women with genetic predisposition for BC and mouse strains with high incidence of BC have been shown to have high 16α-hydroxylation of estrogens [25–27]. Furthermore a parallel increase in *ras* proto-oncogene expression and of estradiol-16α-hydroxylation in human mammary terminal duct-lobular units by a carcinogen has been found [28].

Table 1. Distribution of conjugated metabolites of enterolactone in the culture medium 24 h after the last addition of enterolactone (1 μM) to the medium of HepG2 liver cancer cells in culture[a]

Fraction	%	Fraction	%
Unconjugated	0.12	Monosulfates	77.6
Monoglucuronides	6.29	Disulfates	5.33
Diglucuronides	6.82	Sulfoglucuronides	1.80
In other fractions	2.04		
Total	100.0		

[a]Means of two experiments.

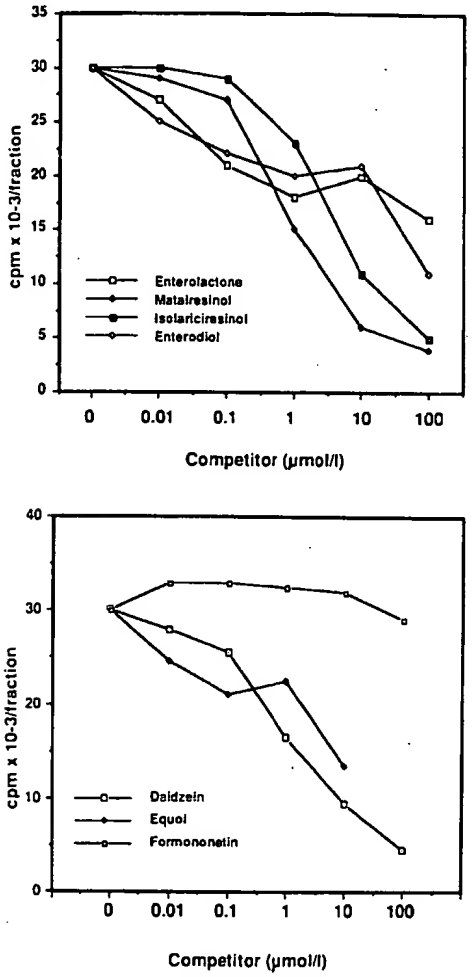

Fig. 3. Competition with [^{3}H]estradiol of lignans (top) and isoflavonoids with regard to rat uterine estrogen nuclear type II binding sites (bioflavonoid receptor).

Several earlier studies by others as well as our own seem to speak against the hypothesis that increased estrogen 16α-hydroxylation is a risk factor for BC, because all low-risk groups, compared to high-risk groups, have relatively more urinary 16α-hydroxylated estrogens, particularly if also the fecal estrogens are included. These observations have recently been discussed [16]. In addition we could previously not observe any increase in 16α-hydroxylated estrogen metabolites in urine of Finnish premenopausal women with BC compared both to omnivorous and vegetarian controls [29].

However, in the present study the old Finnish women showed statistically significant negative correlation between plasma SHBG and urinary 16α-hydroxyestrone and estriol in the whole material ($n = 30$) with the highest values of 16α-hydroxylated estrogens and lowest SHBG values in the women with BC and the omnivorous women. In the same material there was a significant positive correlation between urinary total diphenol excretion and plasma SHBG. All these associations remained statistically different despite elimination of the confounding effect of BMI on the results, although the associations were weaker. It has been shown in many studies that a low SHBG level means a higher metabolic clearance rate and uptake of sex hormones in many tissues including the liver, the principal site of estrogen 16α-hydroxylation. Postmenopausal women with BC have frequently central obesity and low SHBG levels [30–32] and we therefore suggest that some of the *in vivo* results obtained in BC patients showing increased estrogen 16α-hydroxylation may have been at least partly due to a low SHBG in the studied subjects. To our knowledge SHBG was never measured in these studies. In fact it is possible that increased cellular membrane permeability for nonpolar estrogens caused by different mechanisms may also in other tissues lead to increased 16α-hydroxylation.

Estradiol has been found to stimulate the *in vitro* synthesis of SHBG by HepG2 cells in culture, but the concentrations needed for significant increase in production are much higher (0.5–5 μM) than those occurring physiologically [33]. Our experiments show that only 10 times more Enl is needed to show the same stimulation of SHBG formation as that observed for E2. By relating SHBG synthesis to cell number and DNA it could be observed that this was not due to increased cell proliferation but to a true increase in synthesis. This was also confirmed by measuring intracellular SHBG after sonication of the cells.

The question arises whether the concentrations of Enl in the organism, particularly in the portal vein blood, are sufficiently high to have a stimulatory effect on SHBG synthesis. It is well known that estrogens administered orally, compared to parenteral administration, are much more effective in stimulating SHBG synthesis [34]. Enl enters the liver via the portal vein probably in much higher concentrations than those occurring in peripheral plasma. We know very little about the levels of Enl in plasma. Total Enl (free + conjugated) values in 4 women ranged between 0.7 and 5.3 nM [35].

Our own unpublished preliminary observations suggest that the concentrations are much higher in plasma of Finnish women and in vegetarians. We observed total Enl values between 15 and 70 nM and between 20 and over 1000 nM in omnivores and vegetarians, respectively. About 5–30% of the total occurs in the form of unconjugated Enl or in the sulfate form. As found for estrone sulfate, we believe that the sulfates of the lignans can be hydrolyzed at the cell membranes and have biological activity because of the abundance of intracellular sulfatases in the organism. Thus it is very likely that Enl in the free + sulfate form occurs in concentrations at least 10 times higher than those of unconjugated + sulfate-conjugated E2, particularly in the portal vein blood. This makes it very likely that these compounds may be involved in regulation of SHBG levels in plasma in agreement with the positive correlations observed in this and previous studies [5, 9] between excretion of lignans and isoflavonoids in urine and plasma SHBG.

Compared with MCF-7 BC cells [15], the HepG2 cells conjugate Enl as rapidly (Fig. 1 and Table 1), but less monosulfates and higher amounts of glucuronides and disulfates are formed. The monosulfates represented 78% of the total compared to 91% for the MCF-7 cells. Thus our preliminary results in plasma showing considerable amounts of sulfate-conjugated Enl in circulation are in good agreement with the *in vitro* metabolic results obtained with HepG2 cells.

Our results show (Fig. 3) that diphenolic lignans and isoflavonoids compete with E2 for the rat uterine nuclear estrogen type II binding site. These sites seem to constitute a component of the genome which regulates estrogen-stimulated uterine growth [23, 24]. Originally it was observed that some flavonoids like luteolin, quercetin and pelargonin inhibit E2 binding to this receptor and in this way uterine cell growth. They also inhibited growth of MCF-7 cells in culture, and *in vivo* E2 stimulation of immature rat uterus [36]. The structures of these flavonoids are very similar to those of the isoflavonoids. Luteolin, quercetin and pelargonin have to our knowledge not been identified in the human organism. However, Daid, Eq, Enl and End were all found in plasma, saliva and urine of human subjects and Enl, End and Eq in prostatic fluid [37, and unpublished, see above]. Now also Gen, Mat and *O*-Dma have been detected in plasma in our laboratory.

It was suggested that the isoflavonoids and flavonoids may all act synergistically inhibiting cell growth in malignant cells via the type II binding site [16] also called the bioflavonoid receptor [36, 38] or by inhibiting specifically the tyrosine protein kinase [16] the enzyme mediating the activity of many growth factors in the cell.

It is concluded that lignans and isoflavonoids may influence sex hormone metabolism and cancer by influencing plasma SHBG levels resulting in lower uptake and less biological activity of these steroids and by inhibiting growth and proliferation [13–15, 18] of hormone-dependent cancer cells.

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D6

Urinary excretion of lignans and isoflavonoid phytoestrogens in Japanese men and women consuming a traditional Japanese diet[1-4]

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ABSTRACT Epidemiologic studies revealed low mortality in hormone-dependent cancer in Japanese women and men consuming a traditional diet. We previously found that certain diphenolic food components, lignans and isoflavonoids, which are converted to biologically active hormone-like substances by intestinal microflora, may be cancer-protective agents. Therefore, we studied urinary excretion of these compounds (enterolactone, enterodiol, daidzein, equol, and *O*-desmethylangolensin) in 10 women and 9 men in a rural village south of Kyoto, Japan. The subjects consumed a typical low-fat diet with much rice and soy products, fish, and vegetables. An isotope-dilution gas chromatographic–mass spectrometric method was used for the assays. The urinary excretion of lignans was low but that of the isoflavonoids was very high. The excretion of isoflavonoids correlated with soybean-product intake. The low mortality in breast and prostate cancer of Japanese women and men, respectively, may be due to the high intake of soybean products. *Am J Clin Nutr* 1991;54:1093–1100.

KEY WORDS Japanese, diet, urine, lignans, isoflavonoids, enterolactone, enterodiol, daidzein, equol, genistein, *O*-desmethylangolensin, soybean, gas chromatography, mass spectrometry, sex-hormone–binding globulin

Introduction

Mammalian lignans and isoflavonoid phytoestrogens, occurring in all studied animal and human biological fluids and in feces, are diphenolic compounds with molecular weights similar to those of steroid estrogens (1–3). Precursors in plants seem to occur as glycosides (4, 5), and the mammalian compounds are produced from plant lignans and isoflavonoids by intestinal microflora (6–8). Most of the original plant aglycones, such as formononetin, matairesinol, and secoisolariciresinol, occur only in very low concentrations in urine (9, 10). All compounds investigated so far are weakly estrogenic but have shown many other biological activities, producing antiestrogenic (1–3); antiviral (11, 12); and antiproliferative, cytotoxic, and growth-inhibiting effects (3, 13–15). Studies indicate that they most likely stimulate the production of sex-hormone–binding globulin (SHBG) in the liver (2, 14–18) and may in this way significantly influence biological activity of the sex hormones. The higher SHBG values seen in vegetarians (2, 17–19) are probably due to the effect of these diphenolic compounds on liver synthesis of the protein (14). Studies in both young and old women with breast cancer and in various dietary groups indicate that urinary excretion of these compounds is highest in vegetarians and lower in omnivores and breast-cancer patients (2, 18, 20). It was shown that their urinary excretion correlates with the intake of fiber-rich food (2, 17, 18).

Japanese women and women of Japanese origin in Hawaii consuming a diet similar to the original traditional Japanese diet have low breast-cancer incidence and mortality (21–24). Similarly, Japanese men have low mortality with prostate cancer, although autopsy studies have found that the incidence of prostate cancer in Japanese and Western men are similar (25–27). These cancers are sex-hormone dependent and could potentially be influenced both by alterations of sex-hormone metabolism caused by lignans and isoflavonoids or by a direct effect of these compounds on their growth. Because of the associations between diet and these diseases, we decided to study the urinary excretion of lignans and isoflavonoid phytoestrogens in groups of Japanese men and women consuming a traditional diet. A preliminary report was published as an abstract (28).

Subjects and methods

Participants

The subjects participating in this investigation were apparently healthy and were recruited in a small rural village south of Kyoto,

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Japan. Two of the women were found to have hypertension (blood pressure 146/96 and 180/100, respectively). Most of the participants were farmers cultivating tea and rice. Originally 10 men and 10 women volunteered for the study, but 1 man was dropped because his urine volume was not known. Their main work was in agriculture and they consumed mainly their own products. The ages of the men and women were 50.4 ± 18.0 and 46.8 ± 11.5 y, respectively. Height, weight, and body mass index [BMI, in weight (kg)/height $(m)^2$] were, respectively, 160.8 ± 7.8 cm, 58.6 ± 5.8 kg, and 22.7 ± 2.3 for men and 153.1 ± 6.5 cm, 52.9 ± 7.2 kg, and 22.6 ± 3.5 for women. All subjects were within 15% of normal weight.

Collection of samples

Urine was collected for 48 h in plastic bottles containing 2 g ascorbic acid. The bottle was kept in a cool place during collection. The urine was mixed and measured and a sample was frozen as soon as possible and transported to Finland in dry ice for analysis.

Dietary data

The study was carried out in October 1985. Before the survey a nutritionist explained how to weigh the food components and how to write down the results on a form. Most of the food was weighed. Some food, such as bread and milk, was recorded as a piece of bread or cup of milk and the nutritionist estimated the weight of these food items afterwards. Food intake was recorded for 3 d and the nutritionist followed all subjects every day during the survey period. Calculation of the food data was made by an experienced nutritionist using the *Standard Tables of Food Composition in Japan* (29); for fiber calculations the *Food Composition Tables of Dietary Fibers, Minerals, Cholesterol, Fatty Acids* was used (30). The amount of soy sauce in the diet was calculated from the total sodium chloride content of the urine. According to earlier studies Japanese obtain 25.8% of their sodium chloride from soy sauce (31). Soy sauce contains 15% NaCl. The consumption of soy sauce is estimated by using the following formula:

$$\text{Soy sauce} = (\text{amount of NaCl in urine}) \times 0.258/0.15$$

This is the traditional way to estimate soy sauce consumption in Japanese subjects because they do not add any other salt to their food. It is an estimate and not an exact figure and the values were not included in the correlation analyses.

Analytical method

The trivial and systematic names of the compounds measured and discussed are as follows [structures were shown previously (3)]: enterolactone (Enl), *trans*- 2,3-bis[(3-hydroxyphenyl)methyl]-γ-butyrolactone; enterodiol (End), 2,3-*bis*[(3-hydroxyphenyl)-methyl]-butane-1,4-diol; daidzein (Da), 4',7-dihydroxyisoflavone; equol (Eq), 4',7-dihydroxyisoflavan; *O*-desmethylangolensin (*O*-Dma), 1-(2,4-dihydroxyphenyl)-2-(4-hydroxyphenyl)-propan-1-one.

The method used was a modification of a method for determining the estrogen profile in urine by ion-exchange chromatography and capillary gas chromatography–mass spectrometry in the selected ion-monitoring mode (GC-MS-SIM, or GC/MS) (32–34). Originally, estrogens also were determined but because of very low concentrations of some fractions, the amount of urine saved for the purpose was too small and the analyses could not be repeated. Therefore, only the lignan and isoflavonoid values are presented. Only modifications of the method are described.

Protection of the carbonyl functions by ethoximation (necessary only for the estrogens), and extraction with a Sep-Pak C_{18} cartridge (Waters Associates, Milford, MA) were carried out as described (33, 34). The removal of inhibitors of the enzyme hydrolysis by ion-exchange chromatography on a DEAE-Sephadex (Pharmacia Fine Chemicals, Uppsala, Sweden) column in the acetate form was done in a smaller column (0.5 × 3 cm instead of 0.5 × 5 cm). For hydrolysis and purification of the hydrolysate, before evaporation of the last fraction obtained from the above DEAE-Sephadex column, the following deuterated internal standards were added to the eluate: d_6-Enl and -End, d_4-Da and -Eq, and d_5-*O*-Dma (35, 36). This was followed by hydrolysis and Sep-Pak extraction; application of the methanolic extract directly on the QAE-Sephadex A-25 in the acetate form (0.5 × 5 cm); and elution of the estrogens, lignans, and Eq with 4 mL methanol as described. The modification in this step is that *O*-Dma and Da are eluted after this with 4 mL 0.2 mol acetic acid/L in methanol. This fraction is then, after evaporation of the solvent, ready for derivatization (trimethylsilyl ethers) and GC/MS. Selective fractionation of estrogens with vicinal *cis*-hydroxyls was carried out in a borate column with new dimension (0.5 × 3 cm instead of 0.5 × 2.5 cm). Elution of the diphenols was carried out as described and this fraction contains the isoflavan Eq and the two lignans Ent and End.

The two fractions containing lignans and isoflavonoid phytoestrogens and their deuterated internal standards are converted to their trimethylsilyl ether (TMS) derivatives (32) and quantified by GC/MS by using the following ion pairs (mass/charge): Eq, 386/390; Da, 398/402 (and 383/387); End, 410/416; Enl, 442/448; and *O*-Dma, 459/464 (36). The measurements were carried out with a Hewlett-Packard 5995 B GC/MS (Avondale, PA) instrument equipped with a Pascal work station and with an automatic injector.

Urinary excretion of < 0.0025 μmol/d cannot be measured, and between 0.0025 and 0.005 μmol/d the method must be regarded as semiquantitative. The mean values and interassay imprecision for the control pooled-urine sample, measured 59 times in single assays during 1 y, were as follows: Enl, 3.65 μmol/d (CV 7.4%); End, 0.364 μmol/d (CV 11.6%); and Eq, 0.042 μmol/d (CV 9.4%). For Da at a concentration of 0.028 μmol/d, the interassay imprecision is 11.0% (n = 14) and for *O*-Dma at the high concentrations in this study, the interassay imprecision is 8–10% (CV).

The samples were analyzed in two batches and the values for the control sample were almost identical both times and the same as in analyses before and after these two batches.

Statistical methods

The food data are presented as arithmetic means (±SD) and the lignan and phytoestrogen results as arithmetic means (±SD) and geometric means. Geometric means were used when necessary because of skewness of the distribution of the results. The statistical analyses were carried out by using the *StatView* program for Macintosh (Abacus Concepts, Berkeley, CA). The degree of univariate associations between two variables were estimated as Pearson's correlation coefficients (r). The pairs of

TABLE 1
Intake of various food stuffs by the Japanese women and men consuming a traditional Japanese diet*

Nutrient	Women (n = 10)	Men (n = 9)
	g/d	
Rice	578.5 ± 222.5	764.7 ± 240.3
Wheat	59.5 ± 46.0	139.0 ± 113.6
Potato	62.6 ± 30.2	55.2 ± 34.6
Sugar	8.1 ± 7.0	8.1 ± 7.4
Fats	13.1 ± 7.6	12.7 ± 6.9
Pulses and beans	56.5 ± 36.0	40.9 ± 32.0
Fruit	228.2 ± 111.9	146.9 ± 114.0
Green and yellow vegetables	60.6 ± 33.3	55.7 ± 35.2
Other vegetables	139.3 ± 69.3	130.9 ± 77.2
Pickles	32.9 ± 24.9	23.2 ± 21.2
Algae	1.8 ± 2.0	0.7 ± 0.7
Fish	98.7 ± 46.6	113.6 ± 56.5
Meat	37.0 ± 30.1	73.6 ± 58.4
Eggs	38.4 ± 16.6	57.4 ± 30.6
Milk	112.7 ± 131.0	90.9 ± 90.2
Beer	5.1 ± 16.1	454.6 ± 647.1

* $\bar{x}$ ± SD.

adjusted group means for the two groups studied (women and men) were compared by nonpaired t test.

Results

The intake of various types of food are shown in **Table 1**, and **Table 2** shows the results of the calculations with regard to energy;

TABLE 2
Energy intake, intake of various nutrients, and some ratios in the two study groups*

Nutrient	Women (n = 10)	Men (n = 9)
Energy		
(MJ/d)	8.29 ± 1.64	10.79 ± 3.48
(kcal/d)	1973 ± 391	2569 ± 829
Animal protein (g/d)	35.3 ± 13.9	47.8 ± 18.9
Vegetable protein (g/d)	38.2 ± 10.1	45.1 ± 10.6
Total protein (g/d)	73.6 ± 12.2	93.0 ± 28.4
Carbohydrates (g/d)	311.4 ± 77.0	383.3 ± 100.6
Total fat (g/d)	44.4 ± 14.4	51.0 ± 25.9
Total fiber (g/d)	16.9 ± 4.9	15.3 ± 6.0
Animal protein (%)†	47.2 ± 15.9	49.8 ± 7.9
Proteins (%)‡	15.2 ± 2.1	14.6 ± 1.5
Carbohydrates (%)‡	64.6 ± 6.8	68.2 ± 5.1
Fats (%)‡	20.3 ± 5.5	17.2 ± 4.9
Fat (g/kg body wt)	0.86 ± 0.31	0.85 ± 0.37
Fiber		
(mg/J)	2.1 ± 0.7	1.5 ± 0.7
(g/1000 kcal)	8.8 ± 3.0	6.4 ± 3.0
Fiber (g/kg body wt)	0.33 ± 0.10	0.26 ± 0.09
Fat-fiber ratio	2.5 ± 0.9	2.4 ± 0.9

* $\bar{x}$ ± SD.
† Percent of total protein.
‡ Percent of energy.

TABLE 3
Dietary intake of soy products by the two groups studied*

Soy product	Women (n = 10)	Men (n = 9)
	g/d	
Tofu (soybean curd)	25.0 ± 22.9	18.7 ± 28.8
Miso (bean paste)	12.5 ± 6.2	8.5 ± 6.4
Aburaage (fried thin tofu)	2.6 ± 3.6	3.7 ± 4.2
Atuage (fried thick tofu)	4.0 ± 12.7	0.8 ± 2.3
Koridofu (dried soybean curd)	0.37 ± 0.78	0.07 ± 0.2
Fermented soybeans	2.4 ± 4.5	0.9 ± 2.8
Boiled beans	7.7 ± 17.8	6.5 ± 7.8
Soy sauce	22.9 ± 6.1	19.2 ± 4.7
Soy products (sauce excluded)	54.4 ± 34.3	39.2 ± 36.4

* $\bar{x}$ ± SD.

animal and vegetable protein; total proteins, carbohydrates, fats, and fiber; percentage animal protein and percentage protein; and carbohydrate and fat as percent of total calories. Furthermore, we calculated the fat intake per kilogram body weight, fiber intake per J (per 1000 kcal), and the fat-fiber ratio (Table 2). The diet was a low-fat (fat 17.2% and 20.3% of total calories for men and women, respectively), low-animal-protein diet with moderate amounts of fiber and a low fat-fiber ratio, which is typical for the traditional Japanese diet (37).

Table 3 shows the dietary intake of soy products, which were expected to be the most important source of precursors for the urinary isoflavonoids (3).

Table 4 shows the mean excretion values for the two lignans and three isoflavonoid phytoestrogens. The results show a relatively low excretion of enterolactone, a normal excretion for enterodiol, and a very high excretion of isoflavonoid phytoestrogens. The individual results showed large variation, particularly for equol (from 0 to 10.95 μmol/d). For comparison note that the geometric mean values in young omnivorous women living in Helsinki and in Boston for enterolactone, enterodiol, daidzein, equol, and *O*-desmethyl-angolensin were 2.46, 0.20, 0.22, 0.10, 0.03, and 2.05, 0.28, 0.32, 0.07, and 0.03 μmol/d, respectively (2).

TABLE 4
Urinary excretion of lignans and isoflavonoid phytoestrogens in Japanese women and men consuming traditional Japanese diet*

Compound	Women (n = 10)	Men (n = 9)
	μmol/d	
Enterolactone	1.4 ± 1.4 (0.89)	1.1 ± 0.7 (0.89)
Enterodiol	0.7 ± 1.3 (0.41)	0.4 ± 0.3 (0.22)
Total lignans	2.1 ± 2.6 (1.38)	1.5 ± 0.9 (1.13)
Daidzein	2.6 ± 4.0 (2.55)	2.2 ± 2.0 (1.45)
Equol	2.6 ± 4.0 (0.56)	3.0 ± 4.6 (0.54)
O-desmethylangolensin	0.7 ± 0.6 (0.51)	0.2 ± 0.3 (0.11)
Total isoflavonoids	6.9 ± 6.8 (4.73)	3.9 ± 3.3 (2.57)
Total diphenols	9.1 ± 9.3 (6.7)	5.4 ± 4.0 (4.1)

* $\bar{x}$ ± SD (geometric $\bar{x}$).

Table 5 presents a correlation matrix of various food components and urinary excretion of lignans and isoflavonoids in the total material of 19 subjects for whom both food and phytoestrogen data were available.

Discussion

In a previous study of oriental immigrant women from southeast Asia residing in Hawaii (38), the diet was similar to that consumed by the men and women in the rural village in Japan. In the present study the women had a greater energy intake (an additional ~2.1 MJ/d, or 500 kcal/d), which may be due to a physically more active life. However, the percentage intake of calories as fat and the dietary fiber and fat-fiber ratio were very similar to the corresponding values in the previous study. Except for the energy intake the values are very different from those seen in Western societies where the fiber intake is similar but the fat-fiber ratio is much higher. Women living in the Boston area had a fat-fiber ratio of 7.7 for the premenopausal women and 4.6 for the postmenopausal women compared with 2.5 for the women in the present study (39).

With regard to protein intake, expressed as g/d and as percentage of calories, the mean values in the present study were similar and slightly lower, respectively, than those of the immigrants from southwest Asia (38).

Our results are in good agreement with those from an earlier study of 300 female agricultural workers from 18 regions in Japan (37) except for dietary fiber intake, which was much lower (between 5 and 6 g/d) in the women in the earlier study (which may represent crude fiber intake). However, according to the national nutrition survey in Japan, the dietary fiber intake was 22.8 g/d in 1951 and decreased year by year to 17.4 g/d in 1985. These figures are in better agreement with our results obtained in 1985, which show a mean dietary fiber intake in the whole group of ~16 g/d. This latter value is also in good agreement with the value of 13 g/d for nonstarch polysaccharides found by analyses of the Japanese diet in another study (40). On the basis of these investigations and the present investigation, it may be concluded that the amount of dietary fiber in a traditional oriental diet is comparable with that in many Western societies (38–40). We may also conclude that the diet of our subjects was typical for a rural area, where the people to a large extent consume their own products and have a traditional Japanese diet.

The urinary excretion of Enl was, with few exceptions, low in both men and women (Tables 4 and 1A) and was the same as found for the postmenopausal breast-cancer patients in Boston (20). We found a weak correlation between intake of green and yellow vegetables and excretion of Enl and total lignans (Table 5) but no correlation with rice intake. Because these subjects consumed large amounts of rice, it seems justified to conclude that refined rice contains very low amounts, if any, of lignan precursors. There was a better correlation with the intake of soybeans, which thus also may be a source of Enl precursors (Table 5). It is known that soy sauce contains coniferyl alcohol the building block for lignans and lignin (41). The excretion of the lignan End was also found to be associated with the intake of beans and pulses and soy products in general (Table 5).

The excretion of the isoflavonoid phytoestrogens is very high in these Japanese men and women compared with values obtained in women living in Boston (2, 20) and in the Helsinki area (2, 18). The Japanese women in the present study excrete 10 times more Da and 20–30 times more Eq and *O*-Dma than did omnivorous and lactovegetarian women living in the abovementioned two cities. Of the 19 subjects, 47% and 89% excrete micromole amounts of Eq and Da per day, respectively, a phenomenon very rarely seen in subjects consuming a Western diet but seen in subjects consuming a macrobiotic diet (2). The values in an additional study group of nine subjects, including three children (*see* Appendix A), were not significantly different from those in the two main groups (Tables 4 and 1A); they were in fact surprisingly identical. The excretion of matairesinol, the precursor lignan for enterodiol, was very low, but genistein excretion was very high. Genistein is the center of interest in many laboratories because of its very interesting antiproliferative and

TABLE 5
Correlation matrix of various food components and urinary excretion of ligans and isoflavonoids in the whole material (n = 19)

Nutrient	Enterolactone	Enterodiol	Total lignans	Daidzein	Equol	*O*-Desmethylangolensin	Total isoflavonoids	Total diphenols
Green and yellow vegetables	0.525*		0.460*					
Pulses and beans		0.541*	0.492*	0.679†	0.737†	0.617†	0.668†	0.693†
Algae				0.561*			0.450‡	0.430‡
Total fat					0.584†			
Percent fat calories					0.469*			
Fat-fiber ratio					0.507*			
Meat					0.507*			
Soy products (not sauce)		0.481*		0.583†	0.746§	0.601†	0.585†	0.588†
Boiled soybeans	0.758§	0.892§	0.849§	0.632†	0.693§		0.757§	0.801§

* $P < 0.05$.
† $P < 0.01$.
‡ $0.05 < P < 0.10$.
§ $P < 0.001$.

antimitogenic effects (*see* below); genistein showed the highest concentration of all phytoestrogens in urine in these nine subjects. The mean value was almost 6 μmol/d and a value as high as 15.5 μmol/d was observed. Also in this smaller group most variation in the excretion values was found for Eq (from 0.01 to 9.16 μmol/d). In 21.4% of all subjects, equol excretion did not significantly differ from zero: this group included two of the three children; the mother of these two children did not excrete equol in significant amounts.

The low excretion of Enl in the Japanese subjects compared, eg, with Finnish women (2), is most likely due to low intake of grain (whole-grain) products such as bread (2, 17, 18, 42, 43). The precursors of the mammalian lignans seem to be located in the aleuronic layer of the grain close to the fiber (15) but definite evidence for this location has not yet been obtained. The mean End values are similar to those observed in lactovegetarian American and Finnish women and higher than in the omnivorous women from the same countries (2, 20). It is likely that the majority of the lignans in these Japanese subjects is derived from nongrain plant products (pulses and beans), as suggested by the correlations found in Table 5.

Eq excretion correlated positively with the intake of total fat ($P < 0.01$), fat-fiber ratio ($P < 0.05$), and meat ($P < 0.05$) and deviated in this aspect from all the other isoflavonoids. Some subjects are not able to produce Eq at all, as also shown previously for nonJapanese subjects (44). It is possible that those consuming more fat and meat have an intestinal flora more capable of producing Eq from Da, known to occur in large amounts in soybeans (45). Algae may also be a source of isoflavonoids because a positive correlation was found with Da ($r = 0.56$; $P < 0.05$) and total isoflavonoids ($r = 0.45$; $0.05 < P < 0.10$, NS). Algae were suggested to contain factors protective against breast cancer (46).

Lignans and bioflavonoids are candidates for a role as cancer-protective agents (2, 14–16) and as steroid competitors for various enzymes (47). Enl inhibits the aromatase enzyme and competes with the natural substrate androstenedione for the binding site on the cytochrome P450 enzyme (H Adlercreutz, C Bannwart, LE Vickery, et al, unpublished observations, 1985). Phytoestrogens and lignans (48; H Adlercreutz, Y Mousavi, J Clark, et al, unpublished observation, 1987) show interaction with estrogen receptors and flavonoids have antiproliferative effects on the human-breast-carcinoma cell line ZR-75-1 (49). Genistein is a very specific inhibitor of the tyrosine-specific protein kinases (50–55) and platelet-activating-factor–stimulated platelet aggregation, phospholipase C, and tyrosine kinase activity (56). Tyrosine kinase is an important mediator of the effects of some biologically important growth factors such as epidermal growth factor, insulin, platelet-derived growth factor, and insulin-like growth factor on cells. The flavonoids and lignans bind to the type II estrogen-binding sites (15, 57), now also called the bioflavonoid receptor (47, 58), and may in this way regulate by inhibition cell growth and proliferation of hormone-dependent cancers (58). Enzymes metabolizing bioflavonoids and steroids show structurally close similarity (47), indicating that they have the same origin. Furthermore, the isoflavonoid coumestrol complements, as does estradiol, the topography of spaces between base pairs in unwound DNA and simultaneously hydrogen-bond phosphate moieties on opposite strands (59).

One of the most important biological effects of the lignans and isoflavonoids seems to be their stimulation of SHBG synthesis in the liver (2, 14, 16–18). A high SHBG concentration leads to decreased metabolic clearance rate for the sex hormones and lower biological activity. However, Japanese and British women were found to have the same SHBG total-binding capacity, even though Japanese women bound relatively more estradiol to SHBG. This was suggested to be a result of lower affinity of albumin for estradiol in these women (60). It is possible that the phytoestrogens in the high amounts occurring in Japanese women could compete with estradiol for the albumin-binding sites and in this way lead to relatively more binding to SHBG.

SHBG concentrations tend to be lower in breast-cancer patients, particularly in postmenopausal women, and this seems at least partly to be due to diet (15). SHBG-binding capacity was significantly smaller in postmenopausal but not in premenopausal Japanese subjects with breast cancer compared with Japanese control subjects (61), agreeing with our own more recent results in American postmenopausal (43) women. Finnish premenopausal women with breast cancer did not differ in this respect from omnivorous control subjects but they had lower SHBG than did lactovegetarian women (18). Diet seems to be a much more important risk factor for postmenopausal than for premenopausal breast cancer (15). Miso (Japanese soybean paste) (62) or powdered soybean chips (63) (both before and after denaturation of the protease inhibitors) showed a tendency to decrease mammary-tumor formation and growth rate in rat breast-cancer models and soybean diet also reduced breast-tumor incidence in irradiated rats (64). This agrees with the slower average growth rate of postmenopausal breast cancers in Japanese compared with caucasian women in Hawaii (65).

The high concentration of phytoestrogens in the urine of Japanese men could be protective with regard to prostate cancer. Both lignans and isoflavonoids have estrogenic effects in numerous biological systems and may, because of this property, inhibit development of prostatic cancer. It is well known that in Japan and some other Asian countries, despite the same incidence of latent small or noninfiltrative prostatic carcinomas as in Western societies, the mortality is low (25–27). The high exogenous phytoestrogen concentrations could inhibit the growth of the latent carcinomas, postponing their development and making it more likely that the subjects die from some other disease (theory proposed in 1985) (66). Furthermore, the inhibitory effect of genistein on tyrosine-specific protein kinases of certain growth-factor receptors could play an important role. Decreased risk of prostate cancer is seen in Seventh-day Adventist men (67) consuming much beans, lentils, and peas and some dried fruits (rich sources of bioflavonoids) and in men of Japanese ancestry in Hawaii consuming much rice (mainly starch, which has some fiber-like effects in the gut) and tofu (68), supporting the view that these compounds are protective. Recently, Santti's group in Turku, Finland, in a collaborative study with us, observed that dietary soy prevented the development of precancerous changes in a neonatally estrogenized mouse used as a model for prostatic cancer (69), showing that dietary factors may already be important in the fetal and neonatal periods. This study and our observation of high phytoestrogen excretion in urine of children is important because they suggest that these compounds may change the endocrine milieu at the cellular level both in the neonatal period and in prepubertal and adolescent children. Thus, the results cited above and discussed more

extensively elsewhere (14, 15) speak for a role of the diphenols as cancer-protective substances.

It is concluded that Japanese subjects excrete very large amounts of isoflavonoids in urine, mainly genistein, daidzein, and equol, and that the lignan excretion is low. The high excretion of isoflavonoids in urine is related to the intake of soy products in the traditional Japanese diet.

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APPENDIX A

Additional experiments with a modification of the method

The method used in this study was modified further by including the determination of the plant lignan matairesinol [(3R-*trans*)-dihydro-3,4-*bis*[(4-hydroxy-3-methoxy-phenyl)methyl]-2(3H)-furanone)] (intraassay CV = 15.2% and interassay CV = 13.9%) and the isoflavonoid genistein (4',5,7-trihydroxyisoflavane) (intraassay CV = 4.5% and interassay CV = 11.6%) in the assay (1). Because further samples from the present study were not available and because of the recent great interest in genistein we used this new assay in nine other Japanese subjects (three men, three women, and three children) living in Kyoto and consuming a traditional Japanese diet before and during the 24-h urine collection.

TABLE 1A
Urinary excretion of lignans and isoflavonoid phytoestrogens (μmol/d) in nine Japanese subjects (six adults, three children) living in Kyoto and consuming traditional Japanese diet during the urine collection period

Subject, sex, age	Matairesinol	Enterolactone	Enterodiol	Total lignans	Daidzein	Equol	*O*-Desmethylangolensin	Genistein	Total isoflavonoids	Total diphenols
1, M, 41 y	0.010	0.05	0.09	0.15	5.25	6.15	0.12	15.52	27.04	27.20
2, F, 33 y	0.003	2.44	0.15	2.59	3.11	0.01	0.98	4.48	8.58	11.17
3, M, 7 y	0.003	0.07	0.09	0.16	3.23	0.01	0.06	5.66	8.97	9.13
4, M, 6 y	0.006	2.24	0.68	2.93	2.15	0.85	0.51	3.41	6.93	9.85
5, M, 8 y	0.007	0.04	3.39	3.43	3.02	0.02	0.81	4.80	8.64	12.07
6, F, 42 y	0.006	3.25	0.25	3.50	2.20	0.16	1.17	3.55	7.07	10.58
7, M, 38 y	0.012	0.70	0.25	0.96	1.60	0.07	0.40	4.93	6.99	7.95
8, M, 26 y	0.019	1.94	0.18	2.13	3.38	9.16	0.23	7.99	20.76	22.89
9, F, 30 y	0.005	0.62	0.25	0.88	1.25	3.28	0.21	1.85	6.60	7.47
$\bar{x}$	0.010	1.26	0.59	1.86	2.8	2.19	0.50	5.80	11.29	13.15
Geometric $\bar{x}$	0.010	0.50	0.27	1.17	2.58	0.25	0.35	4.91	9.81	11.89

Table 1A shows the individual urinary lignan and isoflavonoid excretion in the additional three men, three women, and three children studied by the new modified procedure, including the results of assays for matairesinol and genistein.

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1. Adlercreutz H, Fotsis T, Bannwart C, Wähälä K, Brunow G, Hase T. Isotope dilution gas chromatographic-mass spectrometric method for the determination of lignans and isoflavonoids in human urine, including identification of genistein. Clin Chim Acta 1991;199:263–78.

D7

ROLE OF PLANT ESTROGENS IN NORMAL AND ESTROGEN-RELATED ALTERED GROWTH OF THE MOUSE PROSTATE

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SUMMARY

The effects of dietary soy on the normal and estrogen-induced neoplastic growth of mouse prostate were studied. Dietary soy was found to have both estrogenic and antiestrogenic properties. In neonatally estrogenized mouse, used as a model for prostatic cancer, dietary soy prevented the development of precancerous changes. These effects may be due to weak estrogens (phytoestrogens) present in soy. The developing prostate is sensitive to estrogen exposure, and it is possible that dietary factors may be important already in the fetal and neonatal period.

INTRODUCTION

There are marked geographic differences in the age-adjusted incidence rates of clinically manifested PC and large latent PC. The incidence rates are high in North American and North European countries and low in Asian countries (1). Diet is the most important and at the same time the most complex and least known factor differentiating the ethnic groups (2).

There are obviously numerous mechanisms which may mediate the effects of diet. It is presumed that the risk of PC is at least partially modified by changes in hormone, particularly estrogen concentrations. The three potential risk factors of prostatic cancer (increasing age, overweight, and diet rich in fat) are all associated with increased estrogen concentration or estrogen/androgen ratio in serum (3,4,5), supporting the hypothesis of androgen-estrogen synergism in the development of PC.

Epidemiologic studies suggest that high vegetable diet could account for the lower risk of PC in Japanese men (6). Both human and animal diet are known to contain various nonsteroidal weakly estrogenic compounds (7). Their importance may lie as much in their ability to antagonize the natural steroid hormones as in their own intrinsic estrogenic activity. In addition to interfering with the prostatic development, these compounds may also affect normal and neoplastic prostatic growth later in life (8).

Dietary estrogens are mainly derived from different edible plants; they may be natural constituents of plants or produced by certain infecting fungus. They are divided in four classes: isoflavonoids, coumestans, mammalian lignans and resorcyclic acid lactones. Isoflavonoids and coumestans (phytoestrogens) are formed in many edible plants, especially soy and other leguminous plants present in substantial amounts for instance in Japanese diet. Mammalian lignans are derived from unrefined grain products as precursors which are activated by intestinal bacteria (9). Resorcyclic acid lactones are produced by fungus, Fusarium, commonly infecting grain and feeds. The relatively good binding affinities of these compounds to estrogen receptor suggest that they may affect estrogen-mediated events.

This study was undertaken to test the possible role of dietary estrogens present in soy in normal and estrogen-related altered growth of the mouse prostate. Soy was chosen because it is rich in plant estrogens and it is the main protein source in standard laboratory animal chow.

MATERIALS AND METHODS

Diets

Standard laboratory chow for mice (R3) and equivalent soyfree chow (R403, soy substituted with casein) were purchased from Ewos, Södertälje, Sweden. Test diets with 6 ppb of diethylstilbestrol (DES) were prepared as described by Thigpen (10).

Collection of urine samples and analysis of phytoestrogens and lignans

Mice were housed in metabolic cages, 3 mice per cage. 24-hour urine samples were collected in plastic jar containing ascorbic acid (1g/l), and after measuring the volumes samples were stored at -70°C. Phytoestrogens and lignans were determined with a capillary gas chromatographic -mass spectrometric procedure as described earlier (9).

Uterus bioassay

Female mice were weaned at the age of 16 days and kept on test diets for 7 days. The animals were sacrificed and weighed, and the uteri were dissected under a microscope and weighed (10).

Dissection of male tissues

Male mice were sacrificed and weighed, and the whole urethroprostatic complexes were removed. For weighing, the tissues were transferred on petri dish containing PBS, and seminal vesicles and prostatic lobes were dissected apart under a microscope as described earlier (11). For histologic preparations the blocks were fixed in Bouin's fixative, dehydrated and embedded in paraffin, and serial 6μm-sections with 400 μm distance were cut through the whole tissue block.

Neonatal estrogenization of the mouse

Neonatal estrogenization was used as a model for prostatic neoplasia. The outbred NMRI male mice are injected with 2 μg of diethylstilbestrol (DES)/pup/day for the first 3 days after birth. This treatment was previously reported to inhibit prostatic growth and function and induce hyperplastic and dysplastic changes at distinct prostatic sites (12). Epithelial cells showed nuclear enlargement and anisokaryosis as well as nuclear hyperchromasia and prominent nucleolei, the changes being typical for dysplasia. These changes are considered to be premalignant.

RESULTS

1. Effect of dietary soy on the excretion of some diet-derived estrogens in mouse urine

When adult male mice were kept on soy-free diet (period 1), they excreted very small amounts of phytoestrogens (desmethylangolensin [*O*-DMA], daidzein, genistein, equol; nmol/l) (Table 1). When they were transferred on standard laboratory chow containing soy for 14 days (period 2), the excretion was 20 - 1000 times higher. The excretion levels returned to previous low levels within 7 days, when the animals were transferred again to soy-free diet (period 3). The excretion of mammalian lignans (enterodiol, enterolactone, matairesinol; nmol/l) present in unrefined grain remained quite unchanged during this experiment.

Table 1.

	O-DMA	DAIDZEIN	GENISTEIN	EQUOL	ENTERO--DIOL	ENTERO-LACTONE	MATAI-RESINOL
SOY- period 1	3	75	35	5	500	227	2
SOY+ period 2	133	1817	1845	5847	1069	455	2
SOY- period 3	2	46	22	8	397	318	1

2. Effect of dietary soy in mouse uterus bioassay - estrogenic and antiestrogenic effects

Dietary soy increased significantly the relative uterine weight (UW/BW = uterine weight/ body weight) in immature female mice whose mothers were either on standard (SOY+) or soy-free (SOY-) diet (Table 2).

Table 2.

DIET	UW/BW (mg/g) (mean ± SEM)	
Fertilization - weaning: SOY+		
18 d - 23 d: SOY+	0.87 ± 0.04	
		p < 0.05
18 d - 23 d: SOY-	0.76 ± 0.03	
Fertilization - weaning: SOY-		
18 d - 23 d: SOY+	0.88 ± 0.07	
		p < 0.05
18 d - 23 d: SOY-	0.79 ± 0.04	

The possible anti-estrogenicity of dietary soy was determined by measuring the ability of to antagonize the effect of dietary DES. The test diets contained 6 ppb of DES, which significantly stimulates the uterine growth. This effect was partially reversed by dietary soy (Table 3).

Table 3.

DIET	UW/BW (mg/g) (mean ± SEM)	
Fertilization - weaning: SOY+		
18 d - 23 d:		
SOY+ +DES	1.01 ± 0.12	
		p < 0.01
18 d - 23 d:		
SOY- +DES	1.49 ± 0.11	
Fertilization - weaning: SOY-		
18 d - 23 d:		
SOY+ +DES	0.78 ± 0.10	
		p < 0.01
18 d - 23 d:		
SOY- +DES	1.26 ± 0.12	

3. Effect of dietary soy on the normal growth of mouse male accessory sex glands - early and long-term effects

Pregnant and nursing animals were kept on standard (soy+) or soy-free (soy-) diet, and after weaning half of the male pups of a litter continued on the same diet and the other half was transferred on different diet. At the age of 2 months the prostatic lobes (ventral prostate=VP, coagulating gland=CG, dorsolateral prostate=DLP), seminal vesicles (=SV) and testes (=T) were weighed (Fig. 1). Before weaning standard diet had inhibitory effect on prostatic growth, compared with soy-free diet. After weaning standard diet stimulated slightly the growth of prostate. The most sensitive parts were coagulating gland and dorsolateral lobe. There were no differences in the testicular weights. No changes were found in the light microscopic structure of prostatic lobes.

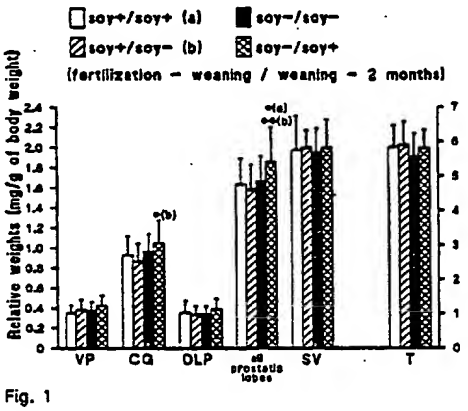

Fig. 1

The weights of prostatic lobes (ventral prostate = VP, coagulating gland = CG, lateral prostate = LP, dorsal prostate = DP) and seminal vesicle (= SV) were significantly higher in animals kept on standard diet from fertilization till the age of 12 months (Fig. 2). There was also significant difference in the testicular weight (= T), suggesting that this long-term stimulatory effect may be indirect. Soy had no effects on the light microscopic structure of prostatic lobes.

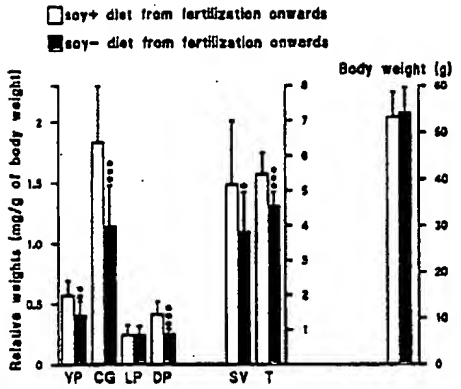

Fig. 2

4. Effect of dietary soy on the growth inhibition in different prostatic lobes induced by neonatal estrogenization

Male mice treated neonatally with DES were kept on standard (soy+) or soy-free (soy-) diet from fertilization till the age of 2 months, when the different prostatic lobes (ventral prostate = VP, coagulating gland = CG, dorsolateral prostate = DLP), seminal vesicles (= SV) and testes (= T) were weighed (Fig. 3). In animals kept on soy-free diet the inhibitory effect of DES on prostatic growth was more severe.

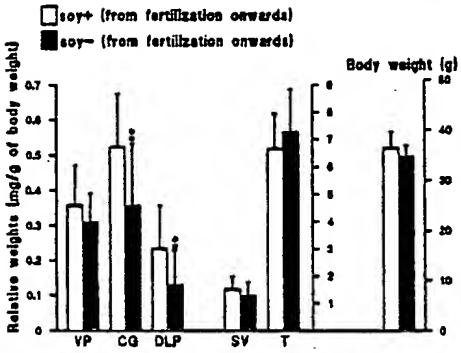

Fig. 3

5. Effect of dietary soy on the development of dysplastic changes in the prostate of neoDES mice

Neonatally DES-treated mice were kept on standard (soy+) or soy-free (soy-) diet from fertilization till the age of 9 months, when the animals were sacrificed. Dysplastic changes were found in all animals kept on soy-free diet. In contrast, only less than half of the animals kept on standard diet showed dysplasia (Table 4). Also the number of dysplastic sites were more numerous in animals on soy-free diet. This suggests that dietary soy may protect from the development of dysplastic changes.

Table 4.

Diet	Number of animals with dysplasia / total number of animals
SOY+	3 / 7 (43 %)
SOY-	7 / 7 (100 %)

CONCLUSIONS

Mice fed standard laboratory chow excreted considerable amounts of weak plant estrogens (isoflavonoids). These compounds were derived from soy. Dietary soy had both estrogenic and antiestrogenic activity in mouse uterus bioassay, which may be due to these plant estrogens. Soy had only marginal effects on the prostatic growth in control animals. In the neonatal period soy slightly inhibited the growth, but in contrast, after weaning it was slightly stimulatory. Whether this reflects direct estrogenic activity on the prostate, remains unclear. Soy was clearly antiestrogenic in animals which were neonatally estrogenized with DES. Moreover, dietary soy prevented the development of dysplastic changes in the prostate of neonatally DES-treated mouse. These effects could be due to phytoestrogens, although other possible mechanisms exist.

Our results suggest that certain type of diet may protect against the development of PC. The relevance of these findings with humans should be considered. Unfortunately, there is very little information on the possible action of dietary estrogens in the human organism. Both men and women are capable of excreting dietary nonsteroidal estrogens in amounts hormonally comparable to the endogenous steroidal estrogens. In a recent study of postmenopausal women, plant foods were found to have significant estrogen-like effect on vaginal cell maturation, which is a sensitive and specific indicator of estrogenicity (13). In women, the urinary excretion of phytoestrogens and lignans, which depends on consumption of vegetables and whole-grain products, correlates positively with plasma SHBG and negatively with percentage free estradiol and testosterone, which could be due to stimulation of SHBG synthesis by weak dietary estrogens. Unlike estradiol, the nonsteroidal estrogens have very low affinity for SHBG in serum (9). This could further enhance their biological potency by increasing the concentration of the free compound available at the target cell.

Phytoestrogens may also interfere with estrogen-mediated processes in the male organism. They may have direct estrogenic effects. However, it is more likely that they act as antiestrogens and prevent exessive estrogenization. The timing of estrogen exposure appears to be critical for the development of prostatic neoplasia, the neonatal period being most sensitive, and it is possible that also the dietary factors may be important already in the fetal and neonatal period. The role of diet should be carefully considered when studies concerning estrogen effects in humans and laboratory animals are carried out.

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(54) Procédé d'obtention d'isoflavones.

(57) On extrait d'une mouture de fèves de soja deux isoflavones particulières, le malonate de génistine et le malonate de daidzine.

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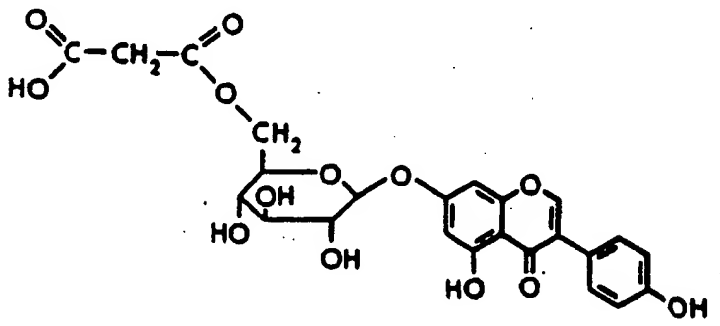
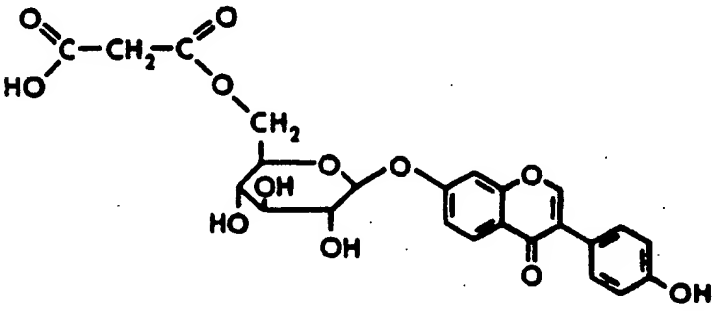
PROCÉDÉ D'OBTENTION D'ISOFLAVONES

La présente invention concerne un procédé d'obtention de deux isoflavones, le malonate de génistine et le malonate de daidzine ainsi qu'une utilisation de ces composés.

On sait, par exemple par l'article de C.M. Francis (J.Sci. Fd Agric. (1973) 24 1235) que les feuilles de trèfle contiennent du malonate de génistine, de formule:



On sait également, par exemple, par le brevet JP 1146894, que l'on peut isoler des racines ou des tiges de Pueraria lobata, le malonate de daidzine, de formule:



La présente invention a pour but de proposer un procédé permettant l'obtention de ces deux isoflavones particulières à partir d'une source alimentaire, à savoir des fèves de soja, d'une manière simple et économique, et avec un bon rendement.

Le procédé, objet de la présente invention, est caractérisé par le fait que l'on extrait une mouture de fèves de soja avec un alcool, on tamponne l'extrait brut obtenu avec une solution aqueuse de tampon, de manière à atteindre un pH de 6-9, on extrait la solution tampon contenant l'extrait brut avec un solvant organique non miscible à l'eau, on récupère la phase aqueuse que l'on acidifie jusqu'à pH 2,5-4, on extrait la phase aqueuse acidifiée avec un solvant organique non miscible à l'eau, on récupère la phase organique que l'on neutralise à pH 6,8-7,2 et l'on sépare les composés résiduels.

Selon le présent procédé, on peut préparer une mouture de fèves de soja, présentant de préférence un diamètre moyen de l'ordre de 0,5-0,8 mm, et un diamètre maximum de 1,0-1,1 mm.

Préalablement à l'extraction, la mouture de fèves de soja peut être dégraissée, afin d'en ôter les matières grasses contenues dans le soja qui pourraient gêner ultérieurement la bonne mise en oeuvre du procédé. Le dégraissage peut être effectué, par exemple, par chauffage sous reflux de la mouture de fèves de soja dans un solvant organique tel que l'hexane ou l'éther de pétrole.

Dans une forme particulière d'exécution, on peut chauffer un mélange comprenant une partie en poids de mouture de fèves de soja avec 4-6 parties en poids de n-hexane, à une température de 60-70 °C, sous une agitation de 70-90 tours par minute et pendant 50-70 minutes, puis laisser refroidir le mélange jusqu'à température ambiante, filtrer puis laver la mouture avec du n-hexane et sécher la mouture dégraissée sous azote pendant 10-15 heures.

On extrait ensuite la mouture éventuellement dégraissée avec un alcool tel que, par exemple le méthanol ou l'éthanol.

Pour ce faire, on peut, par exemple, chauffer sous reflux un mélange comprenant 1 kg de mouture dégraissée et 6 à 8 litres d'une solution aqueuse de méthanol à 70-90%, à une température de 70-90° C, sous une agitation de 70-90 tours par minute et pendant 50-100 minutes, puis filtrer le mélange à chaud et sécher le filtrat, par exemple dans un évaporateur rotatif à 25-50° C et sous pression réduite.

On peut ainsi obtenir un extrait brut se présentant sous forme d'un solide de couleur brune.

On tamponne ensuite l'extrait brut de manière à amener son pH à une valeur de 6-9.

Pour ce faire, on peut mettre l'extrait brut en suspension ou en solution dans une solution de base faible telle qu'une solution aqueuse de $NaCO_3$.

Ceci permet d'améliorer l'extraction venant ensuite, la génistine et la daidzine passant dans la phase organique, et les malonates recherchés restant en phase aqueuse.

On effectue alors une extraction de la solution tampon contenant l'extrait brut, avec un solvant organique non miscible à l'eau tel que, par exemple, le butanol ou l'acétate d'éthyle.

Après extraction, on peut constater que la phase organique contient principalement des isoflavones telles que la génistine et la daidzine, ainsi que d'autres constituants, et que la phase aqueuse contient les composés recherchés ainsi que des impuretés.

On acidifie la phase aqueuse jusqu'à un pH de l'ordre de 2,5-4, par exemple par ajout d'acide chlorhydrique concentré, notamment 5-10 N.

On extrait la phase aqueuse acidifiée avec un solvant organique non miscible à l'eau. On peut choisir, de préférence, un solvant identique à celui utilisé pour l'extraction de la suspension ou solution aqueuse contenant l'extrait brut, c'est-à-dire du butanol ou de l'acétate d'éthyle, ce qui permet d'éviter l'emploi, toujours dangereux, de solvants différents.

On peut ensuite neutraliser la phase organique obtenue en amenant son pH à une valeur de 6,8-7,2, par exemple par ajout de soude, puis sécher ladite phase organique, par exemple en évaporant le solvant dans un évaporateur rotatif à 25-50° C et sous pression réduite.

On obtient ainsi un extrait impur, contenant les composés recherchés, se présentant sous forme d'un solide de couleur brune.

On peut ensuite séparer les composés résiduels de cet extrait, par exemple en effectuant une chromatographie d'adsorption et de filtration de l'extrait impur, ce qui permet également d'en séparer les éventuelles impuretés, telles que la génistine et/ou la daidzine encore présentes.

Cette chromatographie peut être effectuée, par exemple, sur une colonne contenant un gel avec comme éluant un alcool tel que l'éthanol ou un mélange méthanol-eau. On peut obtenir plusieurs fractions contenant des impuretés et plusieurs fractions contenant un mélange des composés recherchés.

On peut alors effectuer une seconde chromatographie des fractions intéressantes, afin de séparer les deux composés recherchés.

Pour ce faire, on peut effectuer une chromatographie en phase inversée en utilisant, par exemple, une colonne polaire contenant notamment des groupes octadécylsilane fixés sur de la silice et un éluant, ou un gradient d'éluants de polarité décroissante, tel que, par exemple, une solution à 10-25% de méthanol, d'éthanol ou d'acétonitrile.

On obtient alors deux fractions, contenant chacune un composé distinct.

Ces fractions peuvent être séchées, par exemple dans un évaporateur-à sec, sous pression réduite et à une température de 25-50° C.

On obtient alors deux composés se présentant sous forme de solides amorphes, l'un de couleur jaune pâle, l'autre de couleur blanche, solubles dans des solvants polaires tels que l'eau, le méthanol ou l'éthanol.

On a constaté que ces deux composés présentaient des propriétés antioxydantes remarquables, et qu'ils pouvaient protéger de l'oxydation par exemple les matières grasses, les vitamines et/ou les oligoéléments contenus dans les produits alimentaires ou cosmétiques.

La présente invention est illustrée plus en détails par l'exemple d'obtention qui suit, ainsi que par les tests d'identification des composés et les tests d'activité qui suivent.

Exemple d'obtention des composés

I) Dégraissage

On ajoute 5 kg de n-hexane à 1 kg d'une mouture de fèves de soja, présentant un diamètre moyen de

0,8 mm.
On chauffe le mélange sous reflux à une température de 65°C et sous agitation constante à 80 tours par minute, pendant 1 heure.
On laisse ensuite refroidir le mélange jusqu'à température ambiante puis on filtre et l'on récupère la mouture qui est mise à sécher sous azote pendant 12 heures.
On mélange à nouveau la mouture sèche à 5 kg de n-hexane et l'on chauffe à 65°C, sous reflux et en maintenant une agitation constante, pendant 1 heure.
On laisse refroidir puis on filtre et on lave la mouture résultante avec 1 litre de n-hexane.
On sèche la mouture sous azote pendant 10 heures. On obtient 0,7 kg de mouture dégraissée de fèves de soja.

ii) Préparation de l'extrait brut

On prépare un mélange comprenant 150 g de mouture dégraissée de fèves de soja telle que préparée selon l'étape i), et 1 litre d'une solution aqueuse de méthanol à 80%.
On chauffe le mélange sous reflux à une température de 80°C pendant 1 heure, en maintenant une agitation constante de 80 tours par minute.
On filtre ensuite à chaud le mélange et l'on réserve le filtrat.
On chauffe à nouveau la mouture avec 1 litre de méthanol à 80%, à 80°C, sous agitation et pendant une heure. On filtre à chaud le mélange et l'on ajoute le filtrat obtenu au filtrat précédent.
On évapore à sec le filtrat total dans un évaporateur rotatif sous pression réduite, à une température de 40°C.
On obtient 32,6 g d'extrait brut se présentant sous forme d'un solide de couleur brune.

iii) Préparation de l'extrait impur

On mélange 32,6 g d'extrait brut tel qu'obtenu selon l'étape ii) à 150 ml d'une solution aqueuse de $NaHCO_3$ 1M.
On ajoute 150 ml de n-butanol, on mélange les deux phases et l'on récupère, par extraction, la phase aqueuse, la phase butanolique contenant principalement de la daidzine et de la génistine.
On acidifie la phase aqueuse jusqu'à pH3 par ajout d'acide chlorhydrique 6,5 N.
On ajoute 150 ml de n-butanol à la phase aqueuse, on mélange et l'on récupère une première phase organique.
On ajoute à nouveau 150 ml de n-butanol à la phase aqueuse, on mélange et l'on récupère une seconde phase organique.
On ajuste le pH des deux phases organiques à 7 par ajout de soude 1N, puis on sèche les deux phases dans un évaporateur rotatif sous pression réduite à 40°C.
On obtient respectivement 2,4 g pour la première phase et 1,2 g pour la seconde phase, d'extrait impur se présentant sous forme d'un solide de couleur brune.

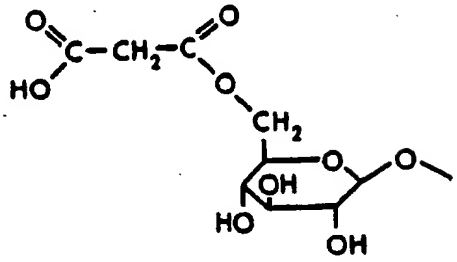
iv) Séparation des composés

On prépare une colonne de chromatographie contenant un gel (Sephadex LH-20), d'un diamètre de 3 cm et d'une hauteur de 50 cm.
On y introduit 2,4 g d'extrait impur tel qu'obtenu selon l'étape iii), dilué avec de l'éthanol.
On élue l'extrait impur avec de l'éthanol, à un débit de 2 ml par minute.
On obtient au total 17 fractions, dont 6 contiennent les malonates de génistine et de daidzine.
On effectue une seconde chromatographie, sur une colonne contenant des groupes octadécylsilane fixés sur de la silice (Lobar RP-18), des 6 fractions intéressantes.
On utilise comme éluant une solution aqueuse d'éthanol à 10%, avec un débit de 2 ml par minute.
On obtient finalement deux fractions contenant chacune un produit différent.
On sèche ces fractions dans un évaporateur sous pression réduite à 40°C.
On obtient 25 mg de malonate de daidzine et 80 mg de malonate de génistine très purs (92-98%) se présentant sous forme de solides amorphes, le premier de couleur blanche et le second de couleur jaune pâle.

Tests d'identification du malonate de génistine

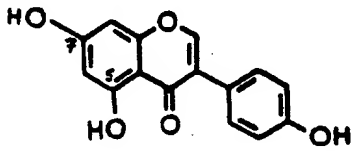
i) Position du goupe sucre

On détermine la position du groupe sucre, de formule:

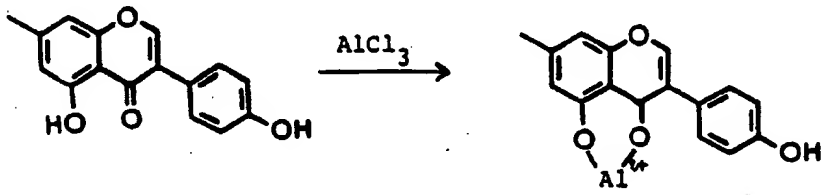


dans le malonate de génistine, à l'aide d'une "shift-reaction" avec du chlorure d'aluminium.

Le groupe OH libre peut se trouver en position 5 ou en position 7, selon la figure ci-après, l'autre position étant occupée par le groupe sucre susmentionné:



Si le groupe OH se trouve en position 5, il va se produire, en présence de chlorure d'aluminium, la formation d'un complexe selon la réaction:



Or le λmax du complexe ainsi formé est supérieur, de 10 à 14 nm environ, au λmax du malonate de génistine. On mesure le λmax du malonate de génistine, dans le méthanol, en présence ou non de chlorure d'aluminium. On obtient les résultats suivants:

. sans $AlCl_3$ λmax = 260,1 nm

. avec $AlCl_3$ λmax = 270,7 nm

Il y a donc eu formation d'un complexe en présence de chlorure d'aluminium; le groupe OH libre est donc en position 5 et le groupe sucre en position 7.

ii) Décomposition en milieu alcalin

On mesure, par chromatographie liquide à haute performance (HPLC) à 228 nm, le temps de rétention de l'acide malonique et du malonate de génistine, tels quels ou en présence d'hydroxyde de sodium.

On observe les résultats suivants:

- acide malonique

on obtient un pic à 2,19 min

- acide malonique + NaOH
on obtient deux pics: un à 2,19 min correspondant à l'acide malonique et un à 5,08 min correspondant au malonate de sodium.
- malonate de génistine
on obtient un pic à 15,17 min
- malonate de génistine + NaOH
on obtient deux pics: un à 12,80 min correspondant à la génistine et un à 5,07 min correspondant au malonate de sodium.
Le malonate de génistine s'est donc décomposé, en présence de soude, en malonate de sodium et en génistine.

iii) Spectre infrarouge

On observe sur le spectre IR du malonate de génistine effectué à l'état solide à la concentration de 1% dans KBr, une bande caractéristique à 1729,2 cm^{-1}, correspondant au groupement carbonyle de l'ester (valeur théorique 1735 ± 10 cm^{-1}).

iv) Spectre de masse

Le spectre de masse a été effectué selon deux méthodes permettant d'obtenir un résultat par défaut ("négatif FAB") et un résultat par excès ("positif FAB").

On obtient les résultats suivants:
- A: pic moléculaire du composé. (malonate de génistine)
- B: pic correspondant au glucoside d'isoflavone (génistine).
C: pic correspondant à l'aglycone de base (génistéine).

Malonate de génistine (poids moléculaire, g)			
	négatif FAB	positif FAB	valeur théorique
A	517	519	518
B	431	433	432
C	269	271	270

v) Spectre RMN

Le spectre de résonance magnétique nucléaire du carbone 13 (RMN) du malonate de génistine dans le diméthylsulfoxyde (DMSO-d6) à 20 °C montre les signaux caractéristiques suivants:

Signal	Multiplicité
45,4 ppm	CH_2
63,1 ppm	CH_2
69,7 ppm	CH
73,0 ppm	CH
74,0 ppm	CH
76,0 ppm	CH
94,3 ppm	CH
99,4 ppm	CH
99,7 ppm	CH
108,3 ppm	C
115,0 ppm	CH (2 carbones)
120,8 ppm	C
122,6 ppm	C
130,0 ppm	CH (2 carbones)
154,0 ppm	CH
157,2 ppm	C
157,7 ppm	C
161,7 ppm	C
162,5 ppm	C
168,4 ppm	C
169,2 ppm	C
179,9 ppm	C

On trouve ainsi 24 atomes de carbone, parmi lesquels 3 carbones carbonyles (acide carboxylique et cétone conjuguée), 14 carbones aromatiques ou oléfiniques (7CH, 7 quaternaires) dont 5 sont O-substitués, un carbone anomérique CH-OH, 4 CH aliphatiques O-substitués, un CH_2 aliphatique O-substitué et un CH_2 aliphatique non O-substitué.
On trouve également 16 protons non interchangeables.

Tests d'identification du malonate de daidzine

i) Décomposition en milieu alcalin

On mesure, par chromatographie liquide à haute performance (HPLC) à 228 nm, le temps de rétention de l'acide malonique et du malonate de daidzine, tels quels ou en présence d'hydroxyde de sodium.
On observe les résultats suivants:
- acide malonique
on obtient un pic à 2,19 min
- acide malonique + NaOH
on obtient deux pics: un à 2,19 min correspondant à l'acide malonique et un à 5,08 min correspondant au malonate de sodium.
- daidzine + NaOH
on obtient un pic à 8,66 min
- malonate de daidzine
on obtient un pic à 13,89 min
- malonate de daidzine + NaOH
on obtient:
a) immédiatement après le mélange, un pic à 11,47 min correspondant à la daidzine et un pic à 8,65 min correspondant à celui obtenu pour la daidzine en présence de NaOH,
b) une heure après le mélange, un pic à 8,65 min correspondant à celui obtenu pour la daidzine en présence de NaOH.

ii) Spectre infrarouge

On observe sur le spectre IR du malonate de daidzine effectué à l'état solide à la concentration de 1% dans KBr, une bande caractéristique à 1734,0 cm^{-1}, correspondant au groupement carbonyle de l'ester (valeur théorique 1735 ± 10 cm^{-1}).

iii) Spectre de masse

Un spectre de masse a été effectué selon deux méthodes permettant d'obtenir un résultat par défaut ("négatif FAB") et un résultant par excès ("positif FAB").

On obtient les résultats suivants:

- A : pic moléculaire du composé (malonate de daidzine).
- B: pic correspondant au glucoside d'isoflavone (daidzine).
- C: pic correspondant à l'aglycone de base (daidzéine)

Malonate de daidzine (poids moléculaire, g)			
	négatif FAB	positif FAB	valeur théorique
A	501	503	502
B	415	417	416
C	253	255	254

iv) Spectre RMN

Le spectre de résonance magnétique nucléaire du carbone 13 (RMN) du malonate de daidzine dans le diméthylsulfoxyde (DMSO-d6) à 20 °C montre les signaux caractéristiques suivants:

Signal	Multiplicité
45,2 ppm	CH_2
63,0 ppm	CH_2
69,7 ppm	CH
73,0 ppm	CH
74,0 ppm	CH
76,1 ppm	CH
99,7 ppm	CH
103,4 ppm	CH
114,9 ppm	CH (2 carbones)
115,3 ppm	CH
118,4 ppm	C
122,0 ppm	C
123,6 ppm	C
126,9 ppm	CH
129,9 ppm	CH (2 carbones)
153,2 ppm	CH
156,8 ppm	C
157,4 ppm	C
161,0 ppm	C
168,3 ppm	C
169,1 ppm	C
174,7 ppm	C

On trouve donc un total de 24 atomes de carbone, parmi lesquels 3 carbones carbonyles (acide carboxylique et cétone conjuguée), 14 carbones aromatiques ou oléfiniques (8CH, 6 quaternaires) dont 4 sont O-substitués, un carbone est anomérique (CH-OH), 4 sont des CH aliphatiques O-substitués, un est un CH_2 aliphatique O-substitué et un est un CH_2 aliphatique non O-substitué.
On a également trouvé 17 protons non interchangeables.

Tests d'activité des composés

i) Analyse qualitative

Il est connu que le β carotène est oxydé en présence d'acide linoléique par irradiation aux ultraviolets (UV). L'oxydation est mise en évidence par la décoloration du β carotène.

On dépose sur une plaque support pour chromatographie sur couche mince, une goutte d'une solution aqueuse de daidzine, de génistine, de malonate de daidzine ou de malonate de génistine.
On vaporise ensuite sur la plaque une solution contenant 100 mg de β carotène et 1 ml d'acide linoléique dans 100 ml de chloroforme. La plaque devient jaune.
On place la plaque sous une source de lumière UV et on l'y laisse jusqu'à l'apparition d'une décoloration.
On observe alors qu'aux endroits où l'on a déposé la génistine et la daidzine, la plaque n'est pas décolorée et présente toujours une coloration jaune.
Ceci est dû au fait que la daidzine et la génistine présentent des propriétés antioxydantes qui peuvent empêcher l'oxydation du β carotène, donc sa décoloration.
On observe aussi que la plaque présente la même coloration jaune aux endroits où l'on a déposé les malonates de génistine et de daidzine.
Ces deux composés présentent donc aussi des propriétés antioxydantes.

ii) Analyse quantitative par spectrophotométrie

On prépare des solutions 0,01 M dans le méthanol de daidzéine, de génistéine, de daidzine, de génistine, de malonate de daidzine et de malonate de génistine.

De manière identique, on prépare code étalons plusieurs solutions d'acide gallique dans le méthanol, dont la concentration varie de 0,2 à 20 gl^{-1}.

On prépare également, comme réactif, une solution contenant 100 mg de β carotène, 1 ml d'acide linoléique et 100 ml de chloroforme.

On prépare des premiers échantillons contenant 20 µl de la solution à mesurer, 60 µl de réactif et 2 ml de méthanol, et des seconds échantillons témoins contenant 20 µl de la solution à mesurer et 2 ml de méthanol.

On mesure, à 450 nm, l'absorbance de l'échantillon par rapport à son échantillon témoin juste après leurs préparations, puis l'on expose les deux échantillons pendant 12 minutes à un rayonnement ultraviolet et l'on effectue à nouveau la mesure de leur absorbance.

On peut alors comparer les résultats obtenus pour les isoflavones aux résultats obtenus pour les différentes solutions d'acide gallique, ce qui permet de définir les équivalences suivantes:

Solution d'isoflavones 0,010 M	Concentration molaire de la solution d'acide gallique présentant le même pouvoir antioxydant
Daidzéine	0,020 M
Daidzine	0,022 M
Malonate de daidzine	0,024 M
Génistéine	0,028 M
Génistine	0,027 M
Malonate de génistine	0,029 M

On constate donc que les solutions 0,010 M de malonate de daidzine ou de génistine ont le même effet que les solutions 0,010 M de daidzine ou de génistine, et que les solutions 0,024 M et 0,029 M d'acide gallique.

iii) Test d'oxydation

En utilisant le test d'oxydation accélérée Rancimat, on détermine les temps d'induction de la graisse de poule stabilisée par addition de malonate de génistine ou de daidzine.

Le test Rancimat® consiste à faire passer de l'air dans un tube de réaction contenant un échantillon de 5 g de matière grasse à 100 °C et à mesurer la conductivité des produits secondaires volatils formés en cours d'oxydation et entraînés avec le courant d'air. On détermine le temps d'induction graphiquement à partir de la courbe enregistrée de la conductivité en fonction du temps par intersection de la tangente à la courbe avec l'axe des temps.

On obtient les résultats suivants pour une graisse de poule contenant 500 ppm de malonate de génistine ou de daidzine ou ne contenant rien (témoin) :

	temps d'induction (h)
témoin	6,95
malonate de génistine 500 ppm	8,10
malonate de daidzine 500 ppm	7,68

L'effet antioxydant du malonate de génistine et du malonate de daidzine est donc bien confirmé.

Revendications

1. Procédé d'obtention d'isoflavones caractérisé par le fait que l'on extrait une mouture de fèves de soja avec un alcool, on tamponne l'extrait brut obtenu avec une solution aqueuse de tampon, de manière à

atteindre un pH de 6-9, on extrait la solution tampon contenant l'extrait brut avec un solvant organique non miscible à l'eau, on récupère la phase aqueuse que l'on acidifie jusqu'à pH 2,5-4, on extrait la phase aqueuse acidifiée avec un solvant organique non miscible à l'eau, on récupère la phase organique que l'on neutralise à pH 6,8-7,2 et l'on sépare les composés résiduels.

2. Procédé selon la revendication 1, caractérisé par le fait que l'on extrait la mouture de fèves de soja avec une solution aqueuse de méthanol à 70-90%, par chauffage à 70-90° C pendant 50-100 minutes.

3. Procédé selon la revendication 1, caractérisé par le fait que le solvant organique non miscible à l'eau est le butanol.

4. Procédé de protection d'un produit alimentaire ou cosmétique de l'oxydation, caractérisé par le fait que l'on incorpore audit produit une quantité efficace de malonate de génistine et/ou de malonate de daidzine.

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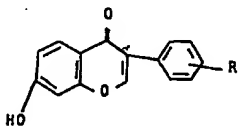
(54) **Method for treatment of osteoporosis.**

(57) A compound of the formula



wherein R is a hydrogen atom or a hydroxy group is effective for prevention or treatment of osteoporosis.

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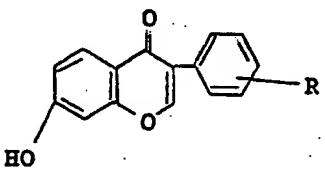
Method for treatment of osteoporosis

This invention relates to a therapeutic means for treatment of osteoporosis.

More particularly, this invention relates to a medicament for prevention or treatment of osteoporosis, which contains a compound of the formula



wherein R is a hydrogen atom or a hydroxy group.

Osteoporosis is a disease condition or illness which occurs frequently in postmenopausal females, particularly those in their sixties, and wherein the quantitative loss of bones progresses beyond a certain limit to thereby present some symptoms or risk manifestations. Among its main clinical manifestations are kyphosis, low back pain, and fractures of femoral neck, lower end of the radius, ribs, upper end of the humerus, etc. While the causative factors are variegated, including endocrine disorder and nutritional disorder, apparently the most important cause is a descreased secretion of estrogen due to hypoovarianism in females during the postmenopausal period. Therefore, of all the therapeutic agents for osteoporosis, the theoretically most effective drugs are estrogen preparations.

However, the estrogens so far available are so strong in effect as to cause side effects such as genital bleeding, mastodynia, hepatic disorder, etc. and, for this reason, have not been used recently on as many occasions as in the past. There are other types of therapeutic agents such as calcitonin, vitamin D and calcium preparations, which however are disadvantageous in that they are either only indefinitely effective or ineffective when administered by the oral route.

The present inventors have found that the compound of the formula (I) exhibits a milder estrogen activity than the conventional estrogens in the oral regimen and does not cause side effects which are produced by these known drugs but cures osteoporosis by stimulating secretion of calcitonin from the thyroid.

The compounds of the formula (I) which are employed in accordance with the present invention are invariably crystalline compounds which are white to pale yellowish brown in color, and are freely soluble in dimethylformamide and chloroform, soluble in ethanol and acetone and practically insoluble in water. When R in the formula (I) is a hydroxy group, it may be present in any position of the phenyl ring.

These compounds can be produced, for example, by cyclizing a 2,4-dihydroxy-phenyl (with or without a hydroxy group on the benzene ring) benzyl ketone to a benzopyran compound, and some of these compounds are known to have capillary vessel stabilizing activity (French Pharmaceutical Patent No. 1065), therapeutic effective for vascular disorders, inflammatory and vitamin-P deficiency disorders (United States Patent No. 3,352,754) or anticonvulsant activity (Japanese Patent Publication No. 32074/1972), but it has not been known that any of the compounds is useful for the treatment of osteoporosis.

As will be apparent from Test Example 5 which appears hereinafter, all of the compounds of the formula (I) are

sparingly toxic. Thus, in the studies in which the compounds were administered orally or subcutaneously to mice or rats at the technically feasible highest doses (5,000 to 10,000 mg/kg), there occurred no death nor toxic symptoms attributable to the compounds.

On the other hand, Test Examples 1 and 2 presented hereinafter show that 7-hydroxy-isoflavone [hereinafter referred to briefly as compound (I)] and 7,4'-dihydroxy-isoflavone [briefly, compound (II)], which are representative species of the compound represented by the formula (I), have mild estrogenic activity which is suited for the treatment of osteoporosis.

Test Example 1

Estrogenic activity of 7-hydroxy-isoflavone in young oophorectomized rats

Sprague-Dawley rats, 33 days old and 11 days after oophorectomy for elimination of endogenous estrogenic activity, were used in groups of 6 to 7 animals. Compound (I) was suspended in a 1% aqueous solution of hydroxypropylcellulose and administered orally for 3 days, while as a representative example of the conventional estrogen drug, estrone was dissolved in sesame oil and administered subcutaneously for 3 days. On the fourth day, each animal was autopsied and its uterine wet weight was recorded. As shown in Table 1, compound (I) at the daily dose levels of 200 mg/kg and 400 mg/kg produced uterine weight increasing effect with a dose-response curve of moderate gradient. In contrast, estrone showed uterine weight increasing effect with a dose-response curve of steep gradient.

Table 1

Compound	Daily dose (mg/kg)	No. of animals	Uterine wet weight (mg ± S.D.)
Compound (I)	0 (control group)	7	35.0 ± 1.0
	6.25	7	32.8 ± 1.1
	12.5	7	33.4 ± 0.9
	25	7	35.1 ± 0.8
	50	7	35.3 ± 1.7
	100	7	35.9 ± 1.0
	200	7	57.9 ± 1.0*
	400	6	70.4 ± 6.7*
Estrone	0.0025	7	101.7 ± 4.6*
	0.005	7	159.8 ± 9.4*
	0.01	7	223.3 ± 12.5*

*: Significant as compared with control group ($P<0.01$)

Test Example 2

Estrogenic activity of 7,4'-dihydroxy-isoflavone in young oophorectomized rats

Sprague-Dawley rats, 33 days old and 11 days after oophorectomy for elimination of endogenous estrogenic activity, were used in groups of 7 animals. Compound (II) was suspended in a 1% aqueous solution of hydroxypropylcellulose and administered orally. As shown in Table 2, compound (II) at the dose level of 400 mg/kg showed mild uterine weight increasing activity.

Table 2

Daily dose of compound (II) (mg/kg)	No. of animals	Uterine wet weight (mg ± S.D.)
0 (control group)	7	31.1 ± 1.1
6.25	7	33.2 ± 0.8
25	7	32.8 ± 1.0
100	7	35.3 ± 1.3
400	7	62.3 ± 6.0*

*: Significant as compared with control group ($P<0.01$)

The following Test Examples 3 and 4 show that the compounds of this invention have bone resorption-inhibiting activity which is effective for the treatment of osteoporosis.

Test Example 3

Bone resorption inhibiting activity of 7-hydroxy-isoflavone and 7,4'-dihydroxy-isoflavone in rat fetal long bone culture.

Determination of bone resorption was performed by the method of Raisz [J. Clin. Invest. 44, 103-116 (1965)]. Thus, a Sprague-Dawley rat on the 19th day of pregnancy was subcutaneously injected with 50 μ Ci of ^{45}Ca (isotope of calcium, $CaCl_2$ solution), and was laparotomized on the following day. The embryos were aseptically taken out, the forelimbs (radius and ulna) were cut off from the trunk under a binocular dissecting microscope, and the connective tissue and cartilage were removed as much as possible to prepare bone samples. Each bone sample was preincubated at 37°C for 24 hours in 0.6 mℓ of the medium containing 2 mg/mℓ of bovine serum albumin in BGJ_b medium (Fitton-Jackson modification) [GIBCO Laboratories, Grand Island, NY 14072 U.S.A.]. Then, the sample was further incubated for 3 days in the same medium as above in which 10 μg/mℓ or 25 μg/mℓ of compound (I) or 10 μg/mℓ of compound (II) had been incorporated. Then, the radioactivity of ^{45}Ca in the medium and that of ^{45}Ca in the bone were measured and the percentage (%) of

^{45}Ca released from the bone into the medium was calculated by the following formula.

Percentage (%) of ^{45}Ca released from bone into medium

$$= \frac{\text{Count of } ^{45}Ca \text{ in medium}}{\text{Count of } ^{45}Ca \text{ in medium + Count of } ^{45}Ca \text{ in bone}} \times 100$$

As control, the bones of the embryos from the same litter were similarly incubated in the absence of compound (I) or (II) for 3 days. The mean ± standard deviation for the six bones per group are shown in Table 3. It is apparent that compounds (I) and (II) suppressed bone resorption.

Table 3

	Concentration of compound	^{45}Ca (%) released	
Control group	0	20.6 ± 3.8	19.9 ± 5.0
Test group 1	Compound (I) 10 µg/mℓ	16.5 ± 2.5*	
Test group 2	Compound (I) 25 µg/mℓ	13.5 ± 2.5*	
Test group 3	Compound (II) 10 µg/mℓ		15.9 ± 1.3**

* : A significant difference from the control group ($P<0.001$)

**: A significant difference from the control group ($P<0.002$)

Test Example 4

Inhibiting activity of 7,4'-dihydroxy-isoflavone to the bone resorption potentiating action of parathyroid hormone in rat fetal long bone culture.

The bone samples prepared in the same manner as Test Example 3 were pre-incubated for 24 hours in the same medium as that prepared in Test Example 3 which contains bovine serum album in BGJ_b medium (Fitton-Jackson modification). Then, in the concomitant presence of PTH (parathyroid hormone, a bone resorption stimulant) and compound (II), the samples

were further incubated for 3 days and the percentage of ^{45}Ca released into the medium was calculated by means of the same formula as that in Test Example 3. The results are shown in Table 4. As control experiments, the same determination was made for a control group using the medium supplemented with PTH alone. It is apparent from Table 4 that compound (II) suppressed PTH-stimulated bone resorption.

Table 4

	Concentration of compound (II)	^{45}Ca (%) released
Control group	0	30.8±4.3
Test group	10 µg/mℓ	23.5±3.4*

*: A significant difference from the control group ($P<0.01$)

Test Example 5

Acute toxicity

Five-week-old ICR mice and 5-week-old Sprague-Dawley rats were respectively used in groups of 10 males and 10 females, and suspensions of compound (I) or compound (II) in olive oil were administered orally [2,500, 5,000 and 10,000 mg/kg of each compound] or subcutaneously [1,250, 2,500 and 5,000 mg/kg]. The animals were kept under observation for 14 days. None of the groups showed deaths nor toxic symptoms attributable to compound (I) or (II) and, therefore, LD_{50} values could not be calculated.

The daily dosage of the compound of the formula (I) according to this invention for human beings is generally about 1 to 50 mg/kg and preferably about 5 to 20 mg/kg for oral administration, and about 200 to 600 mg can be orally taken daily, once a day or, if necessary, in 2 to 3 divided doses. The compounds are preferably formulated into such dosage forms as tablets, capsules, etc. by the established pharmaceutical procedure. Such tablets and capsules can

be prepared using suitable excipients such as lactose, starch, etc., binders such as hydroxypropylcellulose, and lubricants such as magnesium stearate. The tablets may be sugar-coated, if necessary.

The following preparation examples are given to illustrate the invention in further detail and should not be construed as limiting the scope of the invention.

Example 1 Tablets

I)	7-Hydroxy-isoflavone	200 g
II)	Lactose	15 g
III)	Starch	44 g
IV)	Carboxymethylcellulose	10 g
V)	Magnesium stearate	1 g

The above components I) through V) were admixed to prepare 1000 uncoated tablets with a diameter of 8.5 mm.

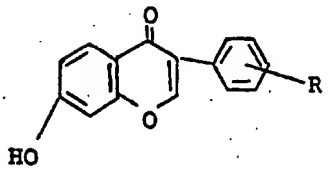
Example 2 Capsules

I)	7,4'-Dihydroxy-isoflavone	200 g
II)	Lactose	40 g
III)	Starch	50 g
IV)	Hydroxypropylcellulose	7 g
V)	Magnesium stearate	3 g

The above components I) through V) were admixed and filled into 1000 No. 1 capsules.

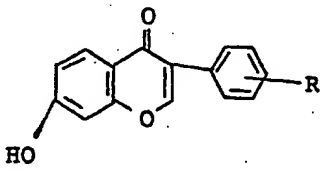
What is claimed is:

1. A compound of the formula



wherein R is a hydrogen atom or a hydroxy group for use in prevention or treatment of osteoporosis.

2. A pharmaceutical composition for prevention or treatment of osteoporosis, which contains an effective amount of a compound of the formula



wherein R is a hydrogen atom or a hydroxy group and a pharmaceutical acceptable carrier, vehicle, lubricant or diluent therefor.

3. A pharmaceutical composition according to claim 2, which is in the form of tablet, capsule, granule, fine granule, powder or syrup.

4 A pharmaceutical composition according to claim 2, wherein the osteoporosis is that caused by decreasing secretion of estrogen due to hypoovarianism.

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Determination of Isoflavones in Soybean Flours, Protein Concentrates, and Isolates

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The individual and total isoflavone content in commercial soybean protein products has been determined by high-performance liquid chromatography. Dehulled, defatted soybean flours contain the following mean isoflavone content (mg/100 g): daidzin, 61.7; glycitein 7-β-glucoside, 12.9; genistin, 119.8; daidzein, 32.8; genistein, 26.7. The same isoflavones were found in soybean protein concentrates and soybean protein isolates but in decreased amounts.

Soybeans contain isoflavones (Naim et al., 1974) that have several known activities, including estrogenic (Drane et al., 1980; Kitts et al., 1980), fungitoxic (Wyman and VanEtten, 1978), and antioxidant (Pratt and Birac, 1979) properties. Because of the ever increasing use of soybean protein products in foods and feeds, it is necessary to know the concentration of these biologically active compounds in various commercial products. Only one report in the literature (Naim et al., 1974) gives any quantitative data on the concentration of isoflavones in soybeans. Therefore, this study has been conducted to determine the amount of these compounds in soybean flours, protein concentrates, and isolates.

MATERIALS AND METHODS

Samples. A dehulled, defatted soybean flour was prepared in the laboratory (Eldridge et al., 1971) from Amsoy soybeans that were grown in 1978. In addition, one sample of commercial soybean meal and eight texturized soybean flours were obtained from various manufacturers. Five commercial samples of soybean protein concentrates (products containing a minimum of 70% protein) were obtained from four manufacturers. Three processors each use a different procedure for the preparation of their concentrates (Circle and Smith, 1972). Five soybean protein isolates (products containing a minimum of 90% protein) were procured from one manufacturer.

Trade names and sources of samples are given in Table I. All samples were ground to pass a 60–80-mesh screen.

Preparation of Extracts. Ground defatted soybean flour was extracted with several solvents to determine the most suitable solvent for dissolving the soybean isoflavones. Solvents investigated were 50%, 80%, and absolute ethanol, 50%, 80%, and absolute methanol, ethyl acetate, and acetonitrile. Refluxing with 80% methanol gave the most reproducible results and maximum extraction.

Table I. Identity of Samples Used in the Study

samples	trade name or description	source[a]
flours		
A	hexane, defatted Amsoy variety, 1978 crop	1
B	Nutrisoy 7B	2
C	unflavored TVP	2
D	Textratein	3
E	Centex 300	4
F	Centex 300 SL	4
G	Centex 400	4
H	Centex 400 SL	4
I	Mira Tex	5
J	Promote III, SL	6
concentrates		
K	Response	4
L	Food protein concentrate	7
M	Pro Con 2000	5
N	Promosoy 100	4
O	GL-301	6
isolates		
P	Edi Pro N	8
Q	Edi Pro A	8
R	Supro 610	8
S	Supro 620	8
T	Supro 710	8

[a] 1, Northern Regional Research Center; 2, Archer-Daniels-Midland Co.; 3, Cargill, Inc.; 4, Central Soya Co.; 5, A. E. Staley Manufacturing Co.; 6, Griffith Laboratories, Inc.; 7, Swift and Co.; 8, Ralston Purina Co.

In the analysis of soybean products, *n*-butyrophenone, which served as an internal standard, was dissolved in 80% aqueous methanol, and an accurate volume was added to the sample. A 1-g sample with 25 mL of 80% aqueous methanol containing the internal standard was heated (boiling) on a steam bath for 4 h, cooled, and filtered through a Type AP prefilter followed by a Type HA, 0.45-μM filter (Millipore Corp., Bedford, MA).

Chromatography. The previously published chromatographic procedure (Eldridge, 1982) was followed, using a linear methanol gradient from 25 to 50% in 20 min followed by an isocratic hold period of at least 30 min. Response factors for the individual isolated glucosides and

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Table II. Effect of Time on Extraction of Isoflavones from Defatted Soybean Meal (Sample A)

		isoflavone content, mg/100 g, as is					
		80% CH_3OH, reflux, 15:1[b]					45:1,[b]
isoflavone	Naim[a]	1 h	2 h	3 h	4 h	5 h	5 h
daidzin	67	44	63	60	65	66	48
glycitein 7-β-glucoside	39	9	11	12	13	13	9
genistin	197	88	118	131	137	144	130
daidzein	tr	7	7	10	11	7	6
glycitein	tr	tr	1	1	tr	tr	tr
genistein	1	2	2	2	2	1	2

[a] Corrected for an assumed 20% oil content. [b] Solvent:sample ratio.

aglycons were determined based on the internal standard. These response factors were used to calculate the isoflavone and isoflavone glucoside composition of various commercial soybean protein products.

RESULTS AND DISCUSSION

Shown in Figure 1 is a typical elution diagram obtained upon chromatographing an 80% methanol extract of soybean flour. This particular elution pattern is for an extract of Centex 400 flour (sample G).

Table II shows results obtained when a single lot (sample A) of hexane-defatted soybean flour was extracted with hot 80% aqueous methanol for various times at different solvent ratios. Also included from Naim et al. (1974), who used GLC, are data that have been corrected to an oil-free basis by assuming 20% oil. As seen in Table II, there is an increase in the extraction of the isoflavone glucosides with time. The largest increase is in genistin, which goes from 88 mg/100 g of meal in 1 h to 144 mg/100 g of meal in 5 h, whereas changing the solvent ratio from 15:1 to 45:1 and extracting for 5 h do not increase the amount of isoflavone glucoside found. The results indicate that the slow extraction of the isoflavone glucosides is not due to limited solubility and that a 4-h extraction appears to be sufficient for extracting the isoflavones from soybean meal.

Table III gives the amounts of isoflavone glucosides and aglycons measured in several commercial defatted soybean flour products. The glucosides daidzin and genistin account for well over 50% of the total isoflavone found in soybean flours. In sample H, these two isoflavone glucosides account for 75% of the total isoflavones measured.

Table IV gives the results of analyzing five different soybean protein concentrates (products which contain a minimum of 70% protein). Concentrates L and O were prepared by aqueous leaching of defatted soybean flours (Circle and Smith, 1972), and the amount of isoflavone measured in the sample approximates the amount of isoflavone measured in soybean flours (Table III). Concentrates K, M, and N, on the other hand, are prepared by

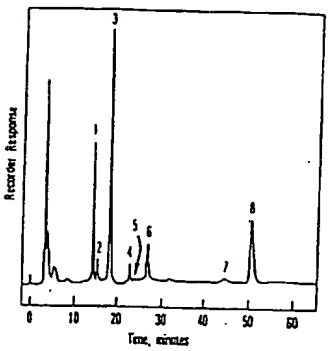

Figure 1. High-performance liquid chromatographic elution diagram of an 80% aqueous methanolic extract of texturized soybean flour. Peaks are (1) daidzin, (2) glycitein 7-β-glucoside, (3) genistin, (4) daidzein, (5) glycitein, (6) genistein, (7) coumesterol, and (8) *n*-butyrophenone.

extracting hexane-defatted soybean meals with aqueous alcohols. This alcohol treatment should remove some of the isoflavones from the meal. The results shown for samples K, M, and N in Table IV indeed show a decrease in the isoflavones.

Table V shows the results obtained when five different soybean protein isolates (products containing at least 90% protein) were analyzed for their isoflavone contents. The majority of the isoflavone measured was genistin, as was observed when soybean flours were analyzed. Although the five soybean protein isolates analyzed are manufactured by different procedures so that the end products have different characteristics, their isoflavone content is fairly constant. About 50% of the total isoflavones in hexane-defatted soybean meal is lost when soybean protein isolate is prepared. The data in Tables III and V show that isoflavone glycosides are preferentially lost in the protein isolation procedure. This decrease in the isoflavone glycosides during protein isolation may be because the glycosides are more soluble than the aglycons in water, which is used for the extraction of soybean protein.

CONCLUSIONS

A high-performance liquid chromatographic procedure has been used to quantitatively measure the isoflavone contents of commercial soybean protein products. The isoflavones in soybeans exist in several forms, i.e., as glucosides, acetyl glucosides, and aglycons. The majority of the isoflavones are present as the glycosides.

In 1980 Drane et al. (1980) showed that rations containing soybean meal prepared for rats caused the uteri of mice to increase in size and concluded soybean meal does have estrogenic activity. Earlier Bickoff et al. (1962) showed that the quantity of genistein and daidzein needed to produce a 25-mg uteri in mice was 8000 and 10 000 μg, respectively. More recent research by Kitts et al. (1980)

Table III. Isoflavone Analysis of Ten Defatted Soy Flours

	mg/100 g, as is, of flour[a]									
isoflavone	A	B	C	D	E	F	G	H	I	J
daidzin	62	61	77	61	49	55	48	77	65	62
glycitein 7-β-glucoside	18	12	22	13	12	13	11	15	6	7
genistin	127	123	146	119	102	58	98	154	142	129
daidzein	48	8	37	46	36	31	17	33	45	27
glycitein	tr	2	3	tr	3	2	tr	tr	tr	tr
genistein	40	4	21	46	27	19	21	26	38	25
total	295	210	306	285	229	178	195	305	296	250

[a] Average of two replicates. Relative standard error per mean is 9.6%. Least significant ratio (0.05 level) of two means is 1.2.

Table IV. Isoflavone Analysis of Five Soybean Protein Concentrates

isoflavone	mg/100 g, as is, of concentrate[a] K	L	M	N	O
daidzin	3	59	9	4	76
glycitein 7-β-glucoside	1	22	2	1	13
genistin	4	124	19	6	191
daidzein	11	20	12	2	11
glycitein	1	tr	tr	1	4
genistein	1	22	1	2	22
total	21	247	43	16	317

[a] Average of two replicates. Relative standard error per mean is 13.7%. Least significant ratio (0.05 level) of two means is 1.5.

Table V. Isoflavone Analysis of Five Soybean Protein Isolates

isoflavone	mg/100 g, as is, of isolate[a] P	Q	R	S	T
daidzin	16	14	23	20	30
glycitein 7-β-glucoside	3	4	4	3	6
genistin	67	59	80	66	55
daidzein	8	12	18	10	21
glycitein	2	1	2	1	3
genistein	22	13	18	5	17
total	118	103	145	105	132

[a] Average of two replicates. Relative standard error per mean is 18.1%. Least significant ratio (0.05 level) of two means is 1.6.

indicates that lesser amounts of genistein may be needed to cause an effect in rat uteri. Our studies show low levels of daidzein and genistein in soybean protein products but large amounts of the isoflavone glucosides. The total isoflavone glucosides and aglycons measured in this study in hexane-defatted soybean meal appears to be approximately 2500 μg/g. Further research is needed to study these soybean constituents as a source of estrogenic response in animals.

ACKNOWLEDGMENT

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Effect of pH on the Extraction and Fractionation of Dry Matter and Crude Protein from Coastal Bermuda Grass and White Clover

John J. Evans

Freeze-dried Coastal Bermuda grass (CBG) and white clover (WC) were extracted at pHs ranging from 4 to 10 and fractionated into four distinct fractions: chloroplastic (CHL), cytoplasmic (CYT), nonprotein nitrogen (NPN), and residue (RES). Dry matter (DM) and crude protein (CP) distributions in the fractions were influenced by pH. At pH 4, the greatest amount of CHL protein was extracted from CBG while the least amount was extracted from WC. At pHs ranging from 6 to 10, the CHL CP extracted remained constant for each forage with WC having twice the CHL CP as CBG. CYT CP extractability exhibited a quadratic effect ($P < 0.001$) due to pH; the pH optima for extraction of CYT proteins occurred at pHs 7 and 8 for CBG and WC, respectively. The amounts of CYT CP extracted from CBG and WC at their optimal pH were equivalent. The NPN fractions increased in CP with increasing pH while the CP in the RES fractions decreased with increasing pH for both forages. In general, the DM distribution paralleled the CP distribution.

The economical production of leaf protein concentrates om forages is desirable since forages can yield more dry atter and crude protein than any other crop (Pirie, 1979). he fractionation of forage proteins into green chloroastic fractions for use in animal feeds and nearly white toplasmic fractions for human use has increased the importance of forages as sources of protein (Subba Rau et al., 1969; Evans et al., 1974; Horigome, 1977; Hanna and Ogden, 1980). Consequently, many different extraction and fractionation procedures have been described (Spencer et al., 1970, 1971; Pirie, 1971; Fishman and Burdick, 1977; Ostrowski, 1979) in an attempt to efficiently extract proteins from different plants. Chloroplastic and cytoplasmic proteins have been separated mainly on the basis of differential heat treatment of the expressed plant juices (Byers, 1967; Lexander et al., 1970; de Fremery et al., 1973; Edwards et al., 1975; Miller et al., 1975). These extraction

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PATENT ABSTRACTS OF JAPAN

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OGAWARA HIROSHI
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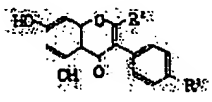
ITO TOKUKI
OGAWARA HIROSHI

(54) IMMUNO-SUPPRESSOR

(57)Abstract:
PURPOSE: To provide an immuno-suppressor containing a specific isoflavone compound as an active component, having low toxicity and excellent immuno- suppressing activity and useful for the remedy and the prevention of relapse of autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus, etc.
CONSTITUTION: The isoflavone compound of formula (R1 is OH or methoxy; R2 is H, carboxyl or ethoxycarbonyl) is used as an immuno-suppressing agent. Concrete examples of the compound are 5,7,4'-trihydroxyisoflavone, 5,7- dihydroxy-4'-methoxyisoflavone-2-carboxylic acid, etc. The compound of formula has excellent immuno-suppressing activity and is useful for the remedy and prevention of relapse of human autoimmune diseases such as chronic active hepatitis, osteoporosis, etc. It is administered orally or parenterally at a dose of usually 200W1,000mg/day.



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[Date of final disposal for application]

⑲日本国特許庁(JP)　⑪特許出願公開

⑫公開特許公報(A)　昭62－106016

⑤Int.Cl.⁴	識別記号	庁内整理番号	㊸公開 昭和62年(1987)5月16日
A 61 K 31/35 // C 07 D 311/34	ABB	6640－4C	審査請求 未請求 発明の数 1 (全3頁)

㊹発明の名称　免疫抑制剤

㉑特　願 昭60－245508
㉒出　願 昭60(1985)11月1日

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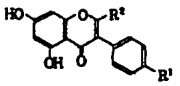
明　細　書

1. 発明の名称

免疫抑制剤

2. 特許請求の範囲

1. 一般式



(式中，R^1は　水酸基または メトキシ基を
R^2は　水素原子，カルボキシ基
またはエトキシカルボニル基
を意味する。)
で示されるイソフラボン化合物を有効成分とする免疫抑制剤

2. 5,7,4'－トリヒドロキシイソフラボンを有効成分とする特許請求の範囲第1項記載の免疫抑制剤

3. 5,7－ジヒドロキシ－4'－メトキシイソフラボン－2－カルボン酸を有効成分とする特許請求の範囲第1項記載の免疫抑制剤

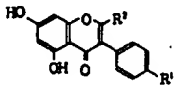
4. 5,7,4'－トリヒドロキシイソフラボン－2－カルボン酸を有効成分とする特許請求の範囲第1項記載の免疫抑制剤

5. 5,7,4'－トリヒドロキシイソフラボン－2－カルボン酸のエチルエステルを有効成分とする特許請求の範囲第1項記載の免疫抑制剤

3. 発明の詳細な説明

(産業上の利用分野)

本発明は，一般式

 　(I)

(式中，R^1は　水酸基 または メトキシ基を
R^2は　水素原子，カルボキシ基
またはエトキシカルボニル基
を意味する。)
で示されるイソフラボン化合物を有効成分とする免疫抑制剤に関する。

（従来の技術）

上記一般式（I）で示される化合物は，R^1が水酸基であるもの全部およびR^1がメトキシ基でR^2がカルボキシ基であるものは，ジャーナル・オブ・ザ・ケミカル・ソサエティー（J. Chem. Soc.）3447頁，1951年および同1852～1859頁，1953年に記載されている化合物である。前者の文献によれば，上記化合物（I）において，R^1が水酸基でR^2が水素原子である化合物はゲニステインと呼ばれており，弱いエストロジェン作用を有することが報告されている。また，後者の文献は，イソフラボン化合物の新しい合成方法を報告しているもので，イソフラボン化合物の薬理作用，殊に免疫抑制作用については全くふれていない。

（発明の作用および効果）

本発明者等は，上記一般式（I）で示される化合物に，新たに免疫抑制作用を見出し，本発明を完成したものである。

以下，化合物（I）の免疫抑制作用，毒性等について説明する。

① 免疫抑制作用

測定方法：

5週令のddY雄マウスの腹腔内に4×10^8個の羊赤血球（日本生物材料センター㈱製）を注射して該マウスを免疫し，その免疫の前後2日間に亘り，1日1回計4回被検化合物（I）の0.5％メチルセルロース（信越化学㈱製）懸濁液を投与した。免疫5日後にマウスを殺して脾臓をとり出し，脾臓中の抗体産生細胞数を検出した。検出は，カニンガム（Cunningham）の方法［カニンガム エー・ジェー，ネーチャー 207巻，1106頁，1965年，(Cunningham A.J., Nature, 207, 1106, (1965)]に従って，被検脾臓細胞をイーグルMEM培地（日水製薬㈱製）で希釈した細胞浮遊液に羊赤血球とモルモット補体を加えて混合し，これをカニンガム チャンバーに入れて両端をパラフィンで封じたのち，37℃のインキュベーター内に90分間静置し，チャンバー内にみられる溶血斑数（イ）（抗体産生細胞数）を数えることにより行う。

対照として，被検化合物非投与マウスについて，同様に溶血斑数（ロ）を数えた。

結　果：

化合物（I）の抑制率

被検化合物	1回投与量(mg/kg)	抗体産生抑生率 (%)
化合物A	5	64.8
	25	41.5
化合物B	5	−
	25	64.3
化合物C	5	67.3
	25	−
化合物D	5	45.3
	25	69.4

注：

（i）抑制率 $= \dfrac{(ロ)-(イ)}{(ロ)} \times 100$

（ii）化合物A：5,7,4'−トリヒドロキシイソフラボン（一般式中，R^1が水酸基でR^2が水素原子の化合物）

（iii）化合物B：5,7−ジヒドロキシ−4'−メトキシイソフラボン−2−カルボン酸（一般式中，R^1がメトキシ基でR^2がカルボキシ基の化合物）

（iv）化合物C：5,7,4'−トリヒドロキシイソフラボン−2−カルボン酸（一般式中，R^1が水酸基でR^2がカルボキシ基の化合物）

（v）化合物D：5,7,4'−トリヒドロキシイソフラボン−2−カルボン酸エチルエステル（一般式中，R^1が水酸基でR^2がエトキシカルボニル基の化合物）

② 毒　性

ddY系マウスを用い，化合物Aは500mg/kgを腹腔内に単回投与し，化合物B，Cは夫々1日25mg/kgを4日間腹腔内に注射し，化合物Dは1日25mg/kgを18日間皮下に注射したが，死亡例はなかった。

以上の測定結果より，化合物(I)は，すぐれた免疫抑制作用を有しており，しかも毒性も低いので，ヒトの免疫疾患，たとえば慢性関節リウマチ，全身性エリセマトーデス，慢性活動性肝炎，骨粗鬆症等の自己免疫疾患の治療および再発予防のための薬剤として有用である。

本発明の免疫抑制剤の臨床投与量は活性成分として，通常成人で1日当り200～1,000㎎であり，これを1～4回に分けて投与する。投与量は患者の状態や年令等，個々の場合に応じて適宜調節される。本発明の免疫抑制剤は単独で治療に供されるほか，他の免疫調節剤又は免疫抑制剤と併用される。他の併用剤としては，たとえばクレスチン，BCG,ビシバニール，レンチナン，ペスタチン,レバミゾール，金製剤，D－ペニシラミン,インターフェロン，インターロイキン，サイモポエチン，γ－グロブリンなどの免疫調節剤，アザチオプリン（イムラン），シクロヘキシミド（エンドキサン），メトトレキセート，サイクロスポリン，ステロイド等の免疫抑制剤が挙げられる。

これらの薬剤と併用する場合の投与量は本発明の薬剤1に対し，併用薬剤0.001～10程度が適当である。

本発明の薬剤の投与は，経口剤（錠剤，カプセル剤，液剤）あるいは非経口剤（直腸投与製剤，注射剤，ペレット）の製剤形態で行なわれる。これ等の製剤は，任意慣用の製剤用担体あるいは賦形剤を通常の方法によって配合された組成物として調製される。この際使用される担体あるいは賦形剤は，一般的に用いられるもので良く，たとえば，錠剤の場合，水，ブドウ糖，乳糖，アラビアゴム，ゼラチン，マンニトール，でん粉ペースト，マグネシウムトリシリケート，タルク，トウモロコシでん粉，ゲラチン，コロイドシリカ，馬鈴薯でん粉，尿素等が利用できる。また液剤は，水性または油性の懸濁液，溶液，シロップ，エリキシル剤であってもよく，これらは通常の方法で調製される。直腸投与のためには，坐剤用組成分として提供され，基剤としては，通常用いられるもの，たとえばポリエチレングリコール，ラノリン，カカオ脂，ウイテプゾル® （ダイナミットノーベル社製）等を使用できる。

特許出願人
山之内製薬株式会社
小河原　宏

D12

FOOD ADDITIVES AND CONTAMINANTS, 1985, VOL. 2, NO. 2, 73–106

Naturally occurring oestrogens in foods—A review

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This review is concerned with the presence of naturally occurring oestrogens in food plants and processed foods. Particular emphasis is placed on isoflavones and coumestans, both of which are true plant oestrogens, and the resorcylic acid lactones, more correctly classified as fungal oestrogens. The metabolism and mode of action of these compounds is discussed and their biological potencies, determined in both *in vivo* and *in vitro* studies, described. Current methods of analysis are indicated and the levels of these oestrogens in food plants, processed foods and feedingstuffs are presented. Botanical, environmental or technological factors affecting the possible intake of plant and fungal oestrogens are mentioned and the hazard associated with such intake is compared with that originating from other dietary or medicinal hormonally active substances. Indications are given of the wide range of common food plants which have been reported to possess oestrogenic (uterotropic) activity, although it is emphasized that in general further work is necessary to substantiate these claims and to confirm the identities of the biologically active principles which have in some cases been proposed. In the concluding section suggestions are made for additional research considered important or necessary in this interesting area.

Introduction

The presence in plants of oestrogens, compounds which induce oestrus in immature animals or interfere with normal reproductive processes, has been known for over half a century. However, the use of plants and plant extracts to control fertility in animals and humans has been recognized since earliest times. In the Orient, for example, the pomegranate has traditional associations with fertility which stretch back over 2000 years. Although many of the plant oestrogens have now been separated, purified and characterized, only occasionally have they been found to be identical with those of animal origin, oestrone (I) and 17β-oestradiol (II) (Hewitt *et al.* 1980) (see figure 1).

In 1954, Bradbury and White listed 53 plants which possessed the capacity to initiate oestrus in animals, but progress in this area was such that only two decades later Farnsworth *et al.* (1975) were able to describe over 300 such plants. In many cases the exact nature of the active principles has not been established but, of the identified compounds, isoflavones and coumestans are the most common. In all, these authors listed 29 plant oestrogens, many of which possessed structural similarity to synthetic diethylstilboestrol (III) (figure 1). Less than half the compounds listed have been reported in plants which are regularly consumed by animals or man. Such plants are listed in table 1 and it may be noted that certain of these, for example legumes and fodder crops, may be consumed in relatively large amounts. Indeed, problems of infertility in livestock (especially sheep) resulting from the grazing of oestrogen-rich pasture or fodders are a serious economic problem in many parts of the world (Hanson *et al.* 1965, Bickoff 1968, Shutt 1976) and have provided the stimulus for much of the

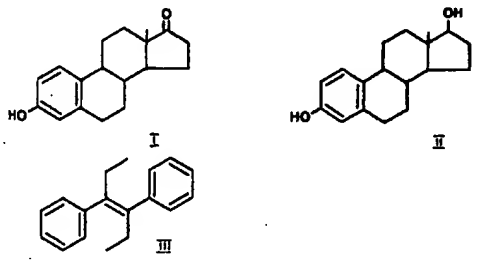


Figure 1. Structures of animal and synthetic oestrogens.

Table 1. Oestrogenic principles of edible plants.

Plant	Common name	Part	Active principle
Avena sativum	oats	seed, meal, sprouts	zearalenone[a] zearalenol[a]
Cicer arietinum	chick pea Bengal gram	seed, seedling	isoflavones isoflavones
Daucus carota var. *sativa*	carrot		3-methyl-6-methoxy-8 -hydroxy,3,4-dihydro isocoumarin[b]
Foeniculum vulgare	fennel	oil	anethole[b]
Glycyrrhiza glabrata	liquorice	root	oestriol, β-sitosterol[b]
Hordeum vulgare	barley	embryo	zearalenone[a]
Humulus lupulus	hops		colupulon[b] lupulon[b] adlupulon[b]
Malus sylvestris	apple	fruit	oestrone
Medicago hispida	toothed medic		isoflavones
Medicago litteralis	barrel medic		coumestrol
Medicago sativa	alfalfa		4-methoxycoumestrol
Oryza sativa	rice	seed, embryo	zearalenone[a] oestrone, oestradiol
Phaseolus vulgaris	French bean	seedling	oestradiol
Phoenix dactylifera	date palm	seed	oestrone
Pimpinella anisum	anise	oil	anethole[b]
Poa pratensis	bluegrass		isoflavones
Prunus avium	cherry	fruit	prunetin
Punica granatum	pomegranate	seed	oestrone
Secale cereale	rye		zearalenone[a]
Sesamum indicum	sesame	meal	zearalenone[a]
Soja max	soya	seed	isoflavones
		sprouts	coumestrol
Sorghum vulgare	sorghum		zearalenone[a]
Triticum vulgare	wheat	flour, seed, germ oil	zearalenone[a]
Trifolium spp.	clovers	leaves	coumestrol
		stems	isoflavones
Vigna sinensis	cowpea		coumestrol
Zea mays	corn		zearalenone[a] zearalenol[a]
	hay		zearalenone[a]

[a] It should be emphasized that zearalenone and zearalenol are not produced by the plant *per se* but may occur on the plant as a result of synthesis by *Fusaria*.

[b] Tentative.

work which has been conducted on plant oestrogens. However, with the exception of those topics of relevance to the presence and effects of plant oestrogens in the human diet, e.g. studies on the analysis and metabolism of oestrogens and of their possible carry-over into the human body via the ingestion of animal products, the role of such compounds in fodders and other animal feedingstuffs will not be considered here.

Additional interest in naturally occurring oestrogens has resulted from the disquiet of scientists, consumers and legislators over the presence in meat and meat products of compounds, such as diethylstilboestrol, designed to improve animal growth and performance (Umberger 1975, McMartin *et al.* 1978). Although the biological activities of such compounds, expressed on a unit weight basis, are very much greater than those of plant oestrogens (see below), under normally regulated conditions their intake into the human body will be very much less. Since any health risk due to dietary factors is a consequence of both biological potency and exposure, there has in recent years been considerable study of plant oestrogens, their metabolism, modes of action and potencies. Such studies have revealed the considerable extent to which genetic, botanical and environmental factors determine the contents of these compounds and also how the processing of the raw plant prior to its consumption can exert similar effects. These studies have, in no small part, benefitted from the development of improved methods of chemical analysis, possessing greatly improved sensitivity and specificity. This paper reviews the more recent advances in these areas and identifies others awaiting additional investigation.

The major plant oestrogens

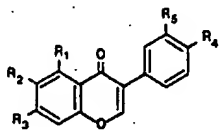
In this review, for convenience, the term 'plant oestrogen' will be used to describe all of the compounds considered in this section, although the resorcylic acid lactones have been referred to elsewhere as *fungal* oestrogens.

The biological effects of plant oestrogens (in the form of the purified compounds or as fresh plants, extracts or processed material) are generally demonstrated by measuring the uterine enlargement of immature female mice or the degree of cornification of the vaginal epithelium. Whereas the former is the more sensitive it lacks the specificity of the latter (Stob 1983); both assays are, however, subject to criticism and misleading results are possible (Emmens 1969).

Examination of table 1 reveals that compounds responsible for the oestrogenic activity mainly fall into three groups, according to their chemical structure. These are (*a*) isoflavones, which in many cases are present in the bound, glycosidic form; (*b*) coumestans; and (*c*) resorcylic acid lactones. A distinction can readily be made between the first two groups and the latter; isoflavones and coumestans are intrinsic plant components, although their levels are dependent upon many factors, including those associated with growth and genetic background. In addition, their levels may also be increased as a direct response to microbial or insect damage. In contrast, the resorcylic acid lactones are products not of the plant *per se*, but of *Fusarium* moulds which are common in the field and flourish in the warm, moist conditions of badly stored grains and other produce. Although other individual compounds possessing oestrogenic activity do occur in food plants, and are considered in the penultimate section of this paper, the major part is concerned with the above groups which are considered separately below.

Isoflavones and isoflavone glucosides

The naturally occurring isoflavones which have been shown to possess oestrogenic activity are (figure 2): daidzein (IV) and genistein (V), their glucosides, daidzin (VI) and



R_1	R_2	R_3	R_4	R_5	
H	H	OH	OH	H	IV
OH	H	OH	OH	H	V
H	H	O-glu	OH	H	VI
OH	H	O-glu	OH	H	VII
H	H	OH	OCH_3	H	VIII
OH	H	OH	OCH_3	H	IX
OH	H	OH	OCH_3	OH	X
OH	H	OCH_3	OH	H	XI
H	H	O-6′acetylglu	OH	H	XII
OH	H	O-6′acetylglu	OH	H	XIII
H	OCH_3	OH	OH	H	XIV
H	OCH_3	O-glu	OH	H	XV

Figure 2. Structures of naturally occurring isoflavones.

genistin (VII), and their 4′-methyl ethers, formononetin (VIII) and biochanin A (IX), respectively; two other active isoflavones, pratensein (X) and prunetin (XI) are of rather limited occurrence. It is possible that other derivatives may also possess uterotropic activity; for example, Japanese workers have isolated the 6′-O-acetyl derivatives of both daidzin and genistin (XII and XIII, respectively) from soyabeans, but they do not appear to have been assayed for their oestrogenic effects (Ohta *et al.* 1979, 1980). It is, however, likely that they are metabolized *in vivo* by ruminants and other animals to daidzin and genistin, or their aglycones. Most of the above isoflavones occur in the intact plant in the bound form, as glucosides, but are readily degraded to the aglycone chemically or enzymically during processing, isolation and analysis. Bound isoflavones in clover and related pastures are readily hydrolysed by endogenous glycosidases when the intact plant is crushed (Beck 1964) and such hydrolysis can also occur in animals, and presumably man, in the absence of the plant enzyme. A large number of isoflavones have been isolated from plant species, but only a small number have been shown to possess oestrogenic activity. Moreover, not all isoflavones isolated from plants known to affect oestrus are active; for example, whilst soyabeans possess daidzein, genistein and their glycosides, they may also contain the uterotropically inactive glycitein (XIV) and glycitein-7β-glucoside (XV) (figure 2) (Naim *et al.* 1973).

The biological potencies of the individual isoflavones vary, but all are much less active than animal or synthetic oestrogens. Thus, although genistein is the most potent isoflavone in terms of its effect on mouse uterus (figure 3), it exhibits only 10^{-5} of the activity of diethylstilboestrol. The relative activities of the individual isoflavones vary with both the species and strain of animal used and with the route of administration. In sheep, biochanin A and genistein were about 20 times less active when introduced

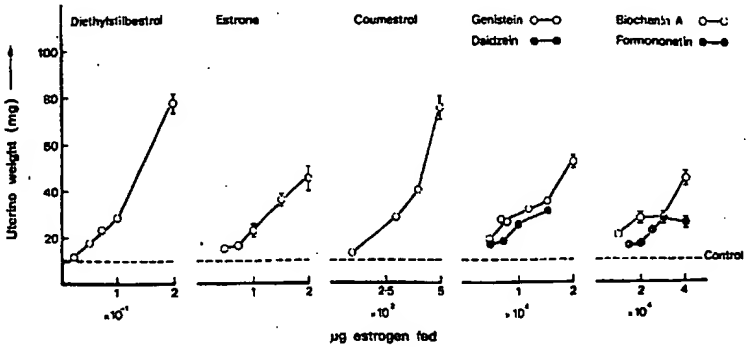


Figure 3. Relative uterotropic potency of diethylstilboestrol, oestrone, coumestrol and isoflavone oestrogens (after Stob 1983).

intraruminally as compared to intramuscular injection, whereas the latter route showed formononetin to be inactive (Braden *et al.* 1976). Genistein was found to be the most active of the isoflavone aglucones tested by oral administration in the mouse (Bickoff *et al.* 1962) and together with its glucoside was equally active when administered subcutaneously (Cheng *et al.* 1955). Differences in responses to other oestrogens between strains of mouse have, however, been reported (Fredericks *et al.* 1981) and recently Farmakalidis and Murphy (1984b) have shown the CD-1 mouse strain to be relatively insensitive to daidzin, genistin and genistein. Comparisons between data arrived at using different strains of mouse are, as the authors point out, thus to be treated with caution. Moreover, there would seem an obvious need to specify, and indeed standardize, the strain of mouse used in the uterotropic assay. Bickoff *et al.* (1962) have demonstrated that dietary isoflavones (daidzein, genistein) possessing a free 4′-hydroxyl group were more uterotropically active in the mouse than their 4′-methyl ethers (formononetin and biochanin A, respectively). The greater potency of genistein compared with daidzein has been attributed to interaction between the OH group and the adjacent carbonyl group of the latter (Bradbury and White 1954). The effect of pratensein is not included in figure 3, but it has been considered to be lower even than formononetin (Wong 1963).

Isoflavones, like the other main groups of plant oestrogens, exhibit an affinity for oestrogen receptor sites (Shutt and Cox 1972, Shutt 1976) and may therefore be considered to function as anti-oestrogens (Martin *et al.* 1978, Verdeal *et al.* 1980). (Anti-oestrogens are thought to exert their effect by decreasing the concentration of cytoplasmic oestrogen receptor and by complexing with the receptor, thus preventing biosynthetic processes associated with tissue development.) The affinities for the binding of genistein to rat, rabbit and sheep uterine cytosol are 1·3, 0·6 and 0·9 respectively (relative to 17β-oestradiol = 100). Other isoflavones are even less active: daidzein exhibits a relative binding affinity of 0·1 and 0·09 for sheep and rat uterine cytosol respectively; biochanin A has an affinity of 0·07 for rat uterine cytosol; and formononetin 0·01 for binding to sheep uterine cytosol (Verdeal and Ryan 1979). The isoflavone metabolites equol, O-desmethylangolensin and angolensin (see below) had relative affinities for sheep uterine cytosol of 0·4, 0·05 and 0·03 respectively (Shutt and Cox 1972).

Based upon the competitive binding to oestrogen receptors in steroid-binding globulins from human breast cancer cells (line MCF-T) the affinities of genistein and formononetin, relative to 17β-oestradiol, are 2 and 0·01 respectively (Martin *et al.* 1978). It seems most likely, as Verdeal and Ryan (1979) have suggested, that transport and metabolic effects are responsible for the apparent discrepancy between the results of the above affinity bioassays and those based upon uterotropic activity. The effects of pure isoflavones in the mouse, rat and sheep are summarized in table 2.

Table 2. Effects of pure isoflavones.[a]

Animal	Compound	Dose	Effect
Mouse	biochanin A	10–40 mg/g diet	uterine hypertrophy
	daidzein	5–15 mg/g diet	uterine hypertrophy
	formononetin	15–40 mg/g diet	uterine hypertrophy
	genistein	5–20 mg/g diet	uterine hypertrophy
	genistein	15 mg/day, diet	infertility, both sexes
	genistein	10 mg injected	displacement of oestradiol from uterine receptors
	genistin	5 mg/day, diet	uterine hypertrophy
	genistin	0·2% diet	infertility, females
	genistin	9–72 mg/day, diet	testes atrophy, depressed growth
Rat	genistein	0·5% diet	testes atrophy, depressed growth
	genistein	0·4 mg, injected	increased protein, phospholipid synthesis in uterus
	genistin	0·5% of diet	testes atrophy, depressed growth
Sheep	biochanin A	1 g, injected	uterine hypertrophy
	formononetin	24 g, injected	uterine hypertrophy
	genestein	1 g, injected	uterine hypertrophy

[a] Full references will be found in Stob (1983), from which this table is taken with permission.

Investigation of the metabolism of isoflavone oestrogens was stimulated by the problem of clover disease in sheep (Bennetts *et al.* 1946). Originally it was assumed that this condition, characterized by a marked loss of fertility, was due to the high levels of isoflavones present in subterranean, and other, clovers. Millington *et al.* (1964) were unable, however, to establish a relationship between the hormonal activity in sheep fed clover and the levels of genistein or biochanin A; a positive relationship was, however, found between the weaker oestrogen, formononetin, and such activity *in vivo*. It is now realized that the reason for this apparently anomalous situation lies in differences in the metabolism of these isoflavones in the digestive tract. Whereas biochanin A and genistein are converted into inactive products, formononetin is metabolized to the isoflavan equol (XVII), and it is this compound in the animal which produces the effect on oestrus (Shutt and Braden 1968). Equol does not, however, appear to be metabolized in the tissues of the sheep (Braden *et al.* 1967). The uterotropic effect of equol is only 10^{-3} that of 17β-oestradiol (Tang and Adams 1980), a potency which is consistent with its relative molar binding affinity to uterine cytosol receptor *in vitro* (Shutt and Cox 1972).

The major pathways which have been elucidated for the metabolism of formononetin (VIII) are shown in figure 4. The primary route, A, involves initial

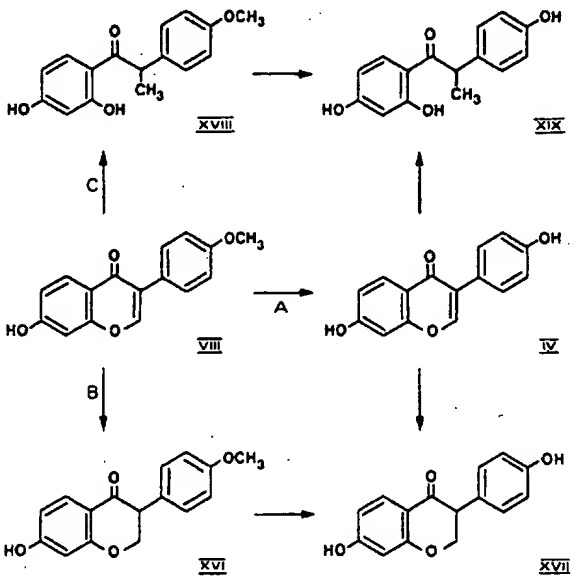


Figure 4. Metabolism of formononetin in the sheep.

demethylation (forming daidzein, IV) and then reduction. A secondary process (B) involves reduction to the 4′-methyl ether of equol (XVI) followed by demethylation. Equol possesses about one half of the affinity for binding to receptor sites of sheep uterine cytosol exhibited by genistein and approximately one quarter of the uterotropic activity of this compound when assayed by intravaginal tetrazolium reduction after oral administration to mice (Shutt and Braden 1968). About 70% of the formononetin ingested by sheep is converted to equol (Shutt *et al.* 1970) and, to a smaller degree, daidzein; in addition, other active metabolites, angolensin (XVIII) and O-desmethylangolensin (XIX) (figure 4, route C) may also be formed (Batterham *et al.* 1971). These compounds are uterotropic in mice and bind to sheep uterine cytosol receptor sites (relative affinities, angolensin 0·03 and O-desmethylangolensin 0·05) (Shutt and Cox 1972). In agreement with the findings of Bickoff *et al.* (1962), referred to above, the oestrogenic activity of the 4′-methyl ether, angolensin, was lower than that of its 4′-desmethyl analogue (Micheli *et al.* 1962).

In marked contrast to the above, biochanin A is metabolized in the sheep via demethylation (to genistein, V) and thence, by ring cleavage (presumably involving the intermediate phenyl-α-methylbenzyl ketone) to the oestrogenically inactive *p*-ethylphenol (XX, figure 5) (Braden *et al.* 1967).

Comparative studies in sheep and cattle revealed the latter to metabolize formononetin more rapidly and also to be more effective in conjugating isoflavones and their metabolites (Braden *et al.* 1971). In sheep the metabolism of biochanin A and genistein in the rumen is initially low but increases significantly over the first few days of grazing on clover and related forages; this is paralleled by a reduction in the hormonal effect of these crops. In marked contrast, the rate of formononetin degradation is not affected by time to any great extent, hence the pasture retains its oestrogenicity

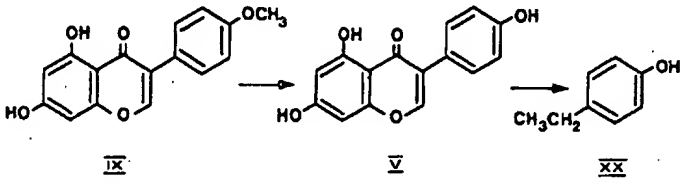


Figure 5. Metabolism of biochanin A in the sheep.

(Lindsay and Francis 1969). Provided that the livestock are removed from such pasture the oestrogenic effects are reversible. However, continued grazing will lead to permanent physiological changes of the reproductive tract (Lindner 1976). Equol has been identified in the urine of goats, rats and hens and in each case was considered to result from dietary isoflavone precursors, rather than being present in the diet *per se*.

Shutt *et al.* (1970) have observed the metabolism of isoflavones in sheep to proceed rapidly; for example, 1 g of biochanin A plus genistein was metabolized in about 90 min. Moreover, the data presented suggested that the initial demethylation (A in figure 4), rather than the reduction, was the rate-limiting step. Equol does not appear to suffer extensive degradation in the rumen, but is readily absorbed therefrom (residence time 1·7 h). There is a suggestion that residence times for isoflavones may be reduced under grazing conditions, a consequence of which may be the less complete metabolism of isoflavones in the rumen, a greater concentration of genistein resulting and/or a decreased production of equol from formononetin (Shutt *et al.* 1970). Consequently the oestrogenic activity, and effect, of such pasture in livestock depends upon the fine balance of isoflavone metabolism *in vivo*.

In contrast to the metabolites of steroidal oestrogens, isoflavones are readily conjugated as glucuronides and excreted. According to Shutt *et al.* (1967) circulating isoflavones are almost exclusively present in the form of biologically inactive glucuronides, although small amounts of the free compounds and their sulphoconjugates, which can yield the free compounds *in vivo*, may also occur. A similar situation has been observed in man (Axelson *et al.* 1982). The plasma content of dietary isoflavones in sheep following the feeding of red or subterranean clovers was maximal 30 min after feeding and thereafter rapidly declined; the content of equol increased from 4 to 10 μg/100 ml plasma between 30 and 150 min after feeding, whilst the measured conjugated equol in plasma was very much higher (300–400 μg/100 ml) and was largely independent of feeding time (Shutt *et al.* 1967).

Equol was first reported in human urine by Axelson *et al.* (1982). Total daily excretion levels of two male subjects were 10·9 and 35·2 μg, whilst those of four female subjects ranged from 10·7 to 43·3 μg. In most cases $\geqslant$ 99·8% of the measured equol was excreted as the glucuronide, but in two subjects 5·7 and 9·9% was bound as the sulphoconjugate. Independently Adlercreutz *et al.* (1982) reported that there was no significant difference in the daily urinary excretion of equol by post-menopausal women who were vegetarians (mean 35·8 μg, range 0–113 μg), omnivores (mean 35·8 μg, range 0–102 μg) or suffering from breast cancer (mean 27·2 μg, range 0–74 μg). The maximum mean daily excretion measured was 565 μg over a three-day period, and at such a level the authors considered that a biological effect might result. Subsequently Bannwart *et al.* (1984) identified both daidzein and equol monoglucuronides in the urine of five female subjects. The levels found in four vegetarian subjects (two pre- and two post-menopausal) were much greater than that measured in the single pre-menopausal,

omnivorous subject (daidzein: average 396·6 μg/l, range 96·0–1108 μg/l compared with 21·6 μg/l; equol: average 4207 μg/l, range 1493–9663 μg/l compared with 46·0 μg/l). The variation in the vegetarian subjects was ascribed to differences in the composition of the diet. Apples, cherries, potatoes, garlic, hops and soya products were mentioned as the most probable sources of dietary oestrogenic compounds. A less important source of these compounds was considered to be products obtained from animals which had been fed oestrogen-containing forage. This seems probable, although only limited data is available upon which to base a judgement. According to Lindner (1967) the levels of such isoflavones accumulating in the adipose tissue of sheep (1 μg/g) was too low to present a serious health hazard. The effects of cooking and/or processing would, moreover, seem likely to reduce this figure further.

Recent work has emphasized the importance of soya as a source of dietary isoflavones (Axelson *et al.* 1984). Two healthy subjects were given 40 g of commercial texturized soya in place of meat, daily for 5 days. Urinary excretion of equol was found to increase 100–1000 fold (figure 6) and traces of daidzein glucuronides were also observed. Quantitatively similar results were observed in rats, approximately 100 μg of equol being excreted per gram of soya flour ingested. The figure for soya oil is much less (5 μg/g), indicating that little, if any, isoflavones are removed from soya during processing of the oil (see below). This result is of interest also since Vague *et al.* (1957) have reported cornification of the vaginal epithelium to occur in post-menstrual women following the administration of 100 g corn or olive oil per day for 10 days. The uterotropic effect of soya meal and soya-based rations in laboratory animals and poultry is well documented (Drane *et al.* 1980).

Setchell *et al.* (1984) have recently shown that certain people excreted little or no equol in the urine when fed 40 g of commercial soya protein daily for 5 days. The reasons for this behaviour are unclear, although it appears to be unrelated to the sex of the subject; the authors suggest that the rate of formation of equol was dependent upon dietary-related factors, such as the composition of the intestinal microflora, the intestinal transit time and variability in the redox level of the large intestine. These

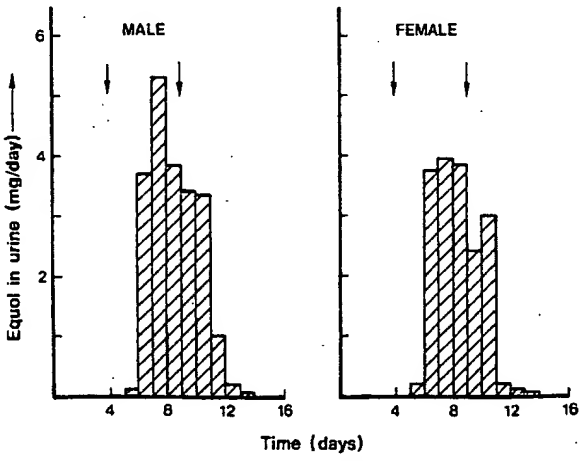


Figure 6. Daily urinary excretion of equol in humans (from Axelson *et al.* 1984). The arrows mark the period over which soya protein, 40 g/day, was fed.

workers also demonstrated that human faecal flora were able to degrade soya-rich broth components (presumably daidzin and daidzein) to equol. As the authors point out, it would be of dubious value to extrapolate the above findings, based upon six subjects, two of whom were obvious non-responders, to the population at large. These results do, however, emphasize the need for further study into the factors affecting phytoestrogen metabolism in man, the metabolic fate of dietary oestrogens in non-responders, and the variation in the rate of phytoestrogen metabolism in larger populations. The latter may in turn lead to the identification of particular 'at-risk' groups within the population at large.

Axelson and Setchell (1981) were unable to determine equol in the urine of germ-free rats fed a commercial, soya-containing ration. Glucosidases capable of converting isoflavone glycosides to the uterotropically active aglucones have been identified in man, as have enzymes which have been shown to carry out the conversion of daidzin to equol (Axelson *et al.* 1984). Although no evidence has yet been presented it would be expected that genistin→genistein→*p*-ethylphenol would represent a similar (but *de*toxifying) metabolic pathway in man.

The improvement of methods for the detection and quantification of isoflavones and their metabolites in plant material and biological samples has been of great importance in the development of an understanding of their chemical and biological properties. Such analysis has been effected by a variety of techniques, including paper chromatography (Markham 1975), thin layer chromatography (Beck 1964), gas chromatography (Naim *et al.* 1974), high-performance liquid chromatography, spectrophotometry, fluorimetry and immunoassay. Gas chromatographic methods, either alone or linked to mass spectrometry, have involved the prior derivatization of the molecules by converting free —OH groups to trimethylsilyl ethers or trifluoroacetyl esters (Naim *et al.* 1973). Gas chromatography-mass spectrometry procedures such as single ion monitoring have allowed very low levels of isoflavone metabolites to be measured (Bannwart *et al.* 1984, Axelson *et al.* 1984), and using conventional gas chromatography procedures the former authors have demonstrated a detection limit of 1 μg equol/24 h urine sample. With such low levels, the isolation, extraction and concentration of the compound(s) of interest from the bulk sample is of paramount importance. The use of reversed-phase silica for preliminary clean-up has proved especially useful for the extraction of isoflavones and equol (and their conjugates) from urine, following which enzyme hydrolysis and ion exchange clean-up processes are employed (Axelson *et al.* 1984, Bannwart *et al.* 1984). Use of DEAE Sephadex (base form) enables a degree of separation between mono- and diphenolic species to be effected (Axelson *et al.* 1982).

The advantage of high-performance liquid chromatography techniques is that the samples can be examined without the need for derivatization; under such conditions both free compounds and conjugates may be analysed directly. Following the original report of Kallela and Saastamoinen (1978), a number of techniques have been described, which almost invariably use reversed-phase systems. Methods developed for the analysis of isoflavones in clover and other fodder crops usually rely upon the facile hydrolysis of the glucosides during plant maceration and extraction such that the isoflavone aglucones are separated and quantified. Petterson and Kiessling (1984) and Sachse (1984) both include chemical hydrolysis prior to sample analysis. Free isoflavones and glucosides are readily determined in soya by high-performance liquid chromatography and, of the methods described, the present authors favour that of Eldridge (1982a) in which all of the likely soya isoflavones are separated, an internal

standard is included and no problems of co-eluting impurities are encountered. The latter severely limits the usefulness of a semi-preparative method for the isolation of daidzin and genistin, reducing the loading capacity to an extent that conventional chromatography (using Sephadex LH20) was of comparable efficiency (Farmakalidis and Murphy 1984a).

The analysis of soyabean (meal) and fractions have almost invariably revealed the presence of daidzin, daidzein, genistin, genistein, glycitin-7β-glucoside and glycitein, the latter two being uterotropically inactive and for this reason not included in table 3. Small amounts of formononetin were also claimed to be present by Shemesh *et al.* (unpublished, cited in Lindner 1976), but details of the method were not given; in the absence of any independent confirmation and bearing in mind the obvious differences between the results of these workers and others (table 3) for the levels of the other isoflavones, this report should be treated with caution. It is generally held that the major proportion of soyabean isoflavones are present as glucosides (table 3), but as has been indicated these are readily degraded by intestinal bacteria prior to metabolism, conjugation and excretion. Bickoff *et al.* (1962) have reported that 8 mg of genistein (or 10 mg of daidzein) was the minimum dose needed to induce a hormonal response in mice; hence the oestrogenic effect of soyabean meal and soyabean-containing commercial rations on poultry and laboratory animals is readily understood, especially when it is further realized that biologically significant levels of coumestrol and its methyl ethers may also be present.

Much research on the isoflavones of pasture and forage crops has demonstrated that many factors (e.g. the physiological age of the plant, its genetic origin, climatic and environmental factors associated with growth) can affect the ultimate content of these compounds in the plant (Bickoff 1968, Rossiter and Beck 1967), and more recent work has shown these factors also to be important in soya. However, additional consideration must be taken of the effect of subsequent processing, especially as it relates to human food ingredients.

Eldridge and Kwolek (1983) have shown that the defatting of full-fat soya does not remove isoflavones or their glucosides, contrary to the earlier claim of Booth *et al.* (1960). Support for this later finding comes from the work of Axelson *et al.* (1984) referred to above. Analysis of soyabean hull (8% by weight), hypocotyl (2%) and cotyledon (90%) fractions revealed isoflavone contents of 10–20 mg/100 g, 1405–1750 mg/100 g and 319–808 mg/100 g respectively. It should be noted that coumestrol is concentrated primarily in the hull and testa portions (Lookhart 1979). Daidzin and glycitin account for more than 95% of the total isoflavone content of the hypocotyl, whereas in the cotyledon the latter is almost absent and genistin predominates. Eldridge (1982b) found that soya protein concentrate (containing 70% protein) prepared by aqueous leaching contained higher levels of isoflavones (247 and 317 mg/100 g) than were present when an aqueous alcohol process was used (16 and 43 mg/100 g). Soya protein isolates, containing 90% protein, although obtained by a variety of unspecified procedures, contained similar isoflavone contents (103–145 mg/100 g), most of which was genistin and genistein. Combined levels of daidzin and daidzein, yielding equol on metabolism, ranged between 24 and 51 mg/100 g. Seo and Morr (1984) found a commercial protein isolate to contain 96 mg isoflavones/100 g. Whilst in general agreement with these findings, Murphy *et al.* (1982) observed the level of isoflavone glucosides in soyabeans to decrease substantially on germination, during protein isolation or when calcium-precipitated tofu was prepared. There appeared, however, to be no corresponding increase in the free forms of these isoflavones. According to György *et al.* (1964)

Table 3. Oestrogenic isoflavone content of soya and its products.

Sample	Daidzin (mg/100 g)	Daidzein (mg/100 g)	Genistin (mg/100 g)	Genistein (mg/100 g)	Formononetin (mg/100 g)	Reference
Soyabean meal	62	48	127	40		Eldridge (1982a)
Soyabean meal	11·7, 0	0, 2·2	74·7, 102·4	4·0, 2·4		Murphy (1982)
Soyabean meal	56·7, 56·1	4·9, 14·5	65·5, 81·3	9·7, 18·7		Pettersson and Kiessling (1984)
Soyabean meal	42	17·8	151	108		Pratt and Birac (1979)
Soyabean flakes	59·6 ± 8	5·6 ± 0·7	215 ± 9	6·7 ± 8		Seo and Morr (1984)
Soyabean flour	48–77	8–48	58–154	4–46		Eldridge (1982)
Soyabean cake		30 ± 5		18·6 ± 2·7	4·3 ± 2	Shemesh *et al.* (in Lindner 1976)
Soyabean flakes	114	2·5	188·5	4·4		Eldridge and Kwolek (1983)
Soya-based animal ration	7		42–45	7		Murphy *et al.* (1982)

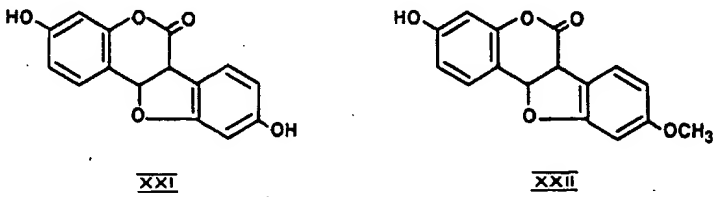
daidzin and genistin are hydrolyzed by *Rhizopus oryzae* during the fermentation of soyabeans to produce tempeh. Defatted soya flakes contained 287 mg isoflavones/100 g (Seo and Morr 1984) and this was decreased by various protein isolation procedures to 203 mg/100 g (acid precipitation), 53 mg/100 g (dialysis), 8·3 mg/100 g (ion exchange) and 6·1 mg/100 g (activated charcoal treatment). A commercial sample of soya protein hydrolysate contained genistein and daidzein contents of 54 and 15·2 mg/100 g, respectively; animal rations containing soya hydrolysates were also observed to possess very low levels of isoflavones (Murphy 1982). Germinated bengal gram (*Cicer arientanum*) was found to contain biochanin A and formononetin at levels of 71 and 77 mg/100 g (Dziedzic and Dick 1982) and 98·6 mg and 44·1 mg/100 g (Sharma 1979a), respectively. The latter worker also identified daidzein (5·1 mg/100 g).

Bartholomew and Ryan (1980) found daidzein, genistein, formononetin and biochanin A all to be non-mutagenic when screened using the *Salmonella*/mammalian microsome assay, the behaviour of the first two compounds being in agreement with the findings of Sugimura *et al.* (1977), and confirmed by Murphy and Glatz (in Murphy 1982).

Isoflavone aglucones have been shown to be responsible in part for the antioxidant activity of soyabeans and their products (György *et al.* 1964, Pratt and Birac 1979, Pratt *et al.* 1981). These compounds also contribute to the astringent and bitter tastes of defatted soyabean (How and Morr 1982) and soy protein products (Huang *et al.* 1981). Soyabean isoflavones possess marked antifungal activity, whereas the glucosides are almost without action (Naim *et al.* 1974). Sharma (1979b) has demonstrated that biochanin A, formononetin and pratensein possess hypolipidaemic activity in the albino rat, but daidzein (and genistein (Ollis 1962)) was inactive. It was considered that this, at least in part, explained the hypocholesterolaemic activity of the black gram and navy bean (Saraswati Devi and Kurup 1972, Hellendoom 1976).

Coumestans

Coumestans possess structures exhibiting close similarity to those of isoflavones to which they are biosynthetically related. A relatively large number of these compounds have been isolated from plants (Wong 1975), but only a few have been shown to possess uterotropic activity. For example, Verdeal and Ryan (1979) list eight coumestans which have been identified in alfalfa, only two of which possess such activity. These compounds, coumestrol (7,12-dihydroxycoumestan, XXI) and 4′-methoxycoumestrol (7-hydroxy-12-methoxycoumestan, XXII) (figure 7) are the most common of this class of oestrogen and have been reported in alfalfa, ladino clover and other fodder crops where their presence is associated with widespread problems of animal performance (Stob 1983). According to Hanson *et al.* (1965), over 90% of the oestrogenic activity of potent dehydrated alfalfa samples was due to its coumestrol content and Lookhart



XXI XXII

Figure 7. Structure of coumestans.

(1980) has found serious oestrogenic effects to result from feeding cattle haylage containing 37 mg coumestrol/kg. Bickoff *et al.* (1960) and others (Micheli *et al.* 1962) have investigated the effect of structural changes in the coumestan molecule on its hormonal activity. Phenolic groups in the 7,12 positions were important; thus the 7-methyl ether and 12-methyl ether (XXII) possessed only 54% and 15%, respectively, of the uterotropic activity of coumestrol itself when administered orally to mice. 7,12-Diacetoxycoumestrol was almost as active as the parent compound when administered in the same manner, presumably reflecting the lability of the acetoxy groupings *in vivo*.

As may be seen from figure 1, the uterotropic potency of coumestrol in the mouse is greater than that of the isoflavones and, as with the latter, variation occurs according to species and means of administration. Braden *et al.* (1967) found coumestrol (administered intraruminally) to be 15 times more active than the most potent isoflavone and it is even more potent when injected intramuscularly. In the mouse, coumestrol is 35 times more active (and its diacetate 24 times more active) than genistein, but still possesses less than 0·03% of the activity of diethylstilboestrol (Bickoff *et al.* 1962). The coumestans, like the isoflavones, bind competitively to mammalian oestrogen receptor sites and are more active when assayed in this manner. The relative binding efficiency (that of 17β-oestradiol = 100) of coumestrol has been reported as 1·4 (rabbit uterine cytosol, Shemesh *et al.* 1972), 4·9 (sheep uterine cytosol, Shutt and Cox 1972), 4·9 (rat uterine cytosol, Verdeal *et al.* 1980) and 19·7 (calf uterine cytosol, Lee *et al.* 1977). When tested in human cancer cell preparations the relative affinity of coumestrol was measured as 9·8 (Martin *et al.* 1978). According to Fredericks *et al.* (1981) coumestrol may exert its effect on fertility *in vivo* by inhibiting follicle stimulating hormone. Little is known about the metabolism of coumestrol; Kelly (1972) has found the compound to be rendered less active in sheep over a period of 7–14 days. Whilst this might be due to the formation of less active metabolites, the chemical nature of which is obscure, more recent work suggests an alternative explanation. Coumestrol is conjugated *in vivo*, but to a rather lower extent than the isoflavones. Thus Kelly and Lindsay (1978) found between 20% and 40% of the total coumestrol in sheep's plasma to be present in the free form (compared to less than 10% in the case of the isoflavones) (Shutt *et al.* 1967). Significantly the concentration of free coumestrol in sheep's plasma remained constant over 16 days, during which time the animals became biologically less sensitive to the oestrogenic effects of this compound. The loss of sensitivity, moreover, appeared to be related to the amount of dietary coumestan and the period of exposure. Further work is needed to clarify the factors underlying these interesting observations. The biological effects of administering coumestrol to animals is shown in table 4.

Coumestrol has been found in a range of plant products commonly consumed by man (table 5). The highest levels were noted in sprouts of alfalfa and, especially, soyabean (Knuckles *et al.* 1976b). Legume sprouts and shoots have in recent years been consumed in increasing amounts by certain sections of the population of the UK and other western countries. It would seem prudent to conduct a more detailed study of the coumestrol (and isoflavone) contents of these materials using modern analytical methods. In the aforementioned work, Knuckles *et al.* used paper chromatography allied to fluorimetric detection and quantification (Knuckles *et al.* 1976a); at the present time, however, the best method of analysis would appear to be high-performance liquid chromatography (Lookhart *et al.* 1978, 1980) using ultraviolet or fluorimetric detection. By judicious choice of mobile and stationary phases it is also possible to monitor isoflavones and coumestrol simultaneously (Pettersson and Kiessling 1984).

Table 4. Effects of pure coumestans and zearalenone.[a]

Animal	Compound	Dose	Effect
Mouse	coumestrol	100–500 μg/g diet	uterine hypertrophy
	coumestrol	500 μg/g diet	antigonadotropic
Rat	coumestrol	1 mg injected, 5 days neonatally	persistent oestrus syndrome
	coumestrol diacetate	125 μg injected	increased protein and phospholipid synthesis in uterus
Sheep	coumestrol	12 mg injected, 1·4 g intraruminally	uterine hypertrophy
Mouse	zearalenone	10 μg/g diet	uterine hypertrophy
		20 μg injected	uterine hypertrophy
Rat	zearalenone	1 mg, oral	uterine hypertrophy
		600 μg topical to skin	uterine hypertrophy
Swine	zearalenone	1–50 mg daily, oral	hypertrophy vulva, vagina, uterus and mammae; metaplasia of cervical epithelial cells
		100 μg/g diet	infertility
		25–100 μg/g diet	infertility, nymphomania, pseudopregnancy, reduced litter size, smaller pigs, malformations, juvenile hyperoestrogenism, probable fetal resorption
Chicken	zearalenone	300–800 μg/g diet	hypertrophy of vent, oviducts and cloacal bursa, eversion of cloaca
Turkey	zearalenone	300–800 μg/g diet	hypertrophy of vent, oviducts and cloacal bursa, eversion of cloaca
Monkey	zearalenone	14 or 56 μg/kg injected	stimulation, LH[b] surge
		14 μg/kg injected	serum LH depression
		400 μg daily, orally for 4 days	serum LH depression

[a] Full references will be found in Stob (1983), from which this table is taken with permission.
[b] LH = luteinizing hormone.

Table 5. Coumestrol content of plant products.[a]

Product	Coumestrol content (μg/100 g dry weight)
Alfalfa sprouts (fresh)	500
Soyabean sprouts (fresh)	7110
Soyabeans (dry)	120
Defatted soyabean meal (dry)	40
Soyabean concentrate	20
Soyabean isolate	60
Frozen green beans	100
Frozen snow beans	60
Frozen green peas	40
Frozen Brussels sprouts	40
Dried red beans	40
Dried split peas	30
Frozen spinach leaf	10

[a] Data from Knuckles *et al.* (1976) with permission.

The coumestrol content of plant material has been observed to vary with a variety of factors (Bickoff *et al.* 1969). For example, Hanson *et al.* (1965) have shown that of alfalfa to be affected, to various degrees, by variety, stage of growth, cutting, the year and location and, to a significant degree, by the presence of disease. Coumestrol has been observed to accumulate in alfalfa and other legumes following insect (Loper 1968) or fungal attack (Loper 1968, Loper and Hanson 1964, Stuthman *et al.* 1966, Loper *et al.* 1967). According to Sherwood *et al.* (1970) coumestrol was not translocated from the infected area to other parts of the plant. Whereas coumestrol in undamaged, non-infected plants was metabolized via the isoflavone pathway (Grisebach and Barz 1963, 1964), the origins of the coumestrol biosynthesized as a result of such insect or fungal damage is unknown.

Concern over the presence of coumestans in alfalfa and ladino clover has resulted from the reduced reproductive performance of animals maintained on such fodder (Hanson *et al.* 1965, Bickoff *et al.* 1969) and both breeding programmes and improved husbandry practices have been initiated to reduce the extent of the problem. Of the latter, treatment with agrochemicals can minimize the pest and fungal attack which results in accumulation of coumestrols and other plant phenolics; moreover, the intake of coumestrol by animals can also be reduced by the feeding of immature plants in which the coumestrol content is known to be lower than in the mature plant. In the absence of any information concerning the amount, if any, of coumestans which enter the human body indirectly via the residues in animal products and milk obtained from livestock grazing on oestrogenic pasture, concern over the intake of these compounds by man is centred mainly upon their presence in common food plants (table 4), vegetable protein and 'health' products.

Leaf protein concentrate has been suggested as a source of protein for humans, and methods have been described for its preparation from alfalfa (Kohler *et al.* 1968, Edwards *et al.* 1975). The effect of such processing on the coumestrol content has been examined by Knuckles *et al.* (1976b). Relatively little of the original coumestan content of the alfalfa (11–118 mg/kg) was removed in the solubles during the early stages of the processing. Protein concentrates possessing 9–14 mg coumestrol/kg were obtained by commercial-type processing in which heat coagulation and washing was carried out under acid conditions (pH 4·5–6·5), whereas if the medium was kept alkaline (pH 8·5–9·5) the coumestrol content was much lower (3 mg/kg) due to the greater solubility of the oestrogen under these conditions. Diafiltered alfalfa leaf protein concentrate possessed a coumestrol content of only 0·4 mg/kg (measured as freeze-dried powder). Since the coumestrol content of diseased or damaged alfalfa leaves may exceed 1000 mg/kg, i.e. 10–100 times that of undamaged tissue, it is clearly important that the quality of the materials selected for processing be maintained as high as possible.

Alfalfa and other leguminous products have been widely marketed in recent years as health foods, tonics and supplements. Recently, Elakovich and Hampton (1984) have analysed commercial alfalfa tablets and found these to contain 20–194 μg coumestrol/g, equivalent on a daily dosage basis to 1–2 mg of coumestrol. The effect of long-term exposure to such levels (together with that of any isoflavone oestrogens which may also be present) cannot yet be ascertained. However, this work clearly points to the desirability of monitoring the contents of physiologically active substances in health products since the 'recommended' doses (if stated) are frequently exceeded and the products may not be covered by the same legislative controls as foods and feeding-stuffs.

Coumestrol has been observed to possess tumour-promoting activity similar to that of 17β-oestradiol and diethylstilboestrol for dimethylbenzanthracene-induced rat mammary tumours (Verdeal *et al.* 1980). However, Bartholomew and Ryan (1980) have reported this compound to be non-mutagenic in the Ames test. Both coumestrol and its 4′-methyl ether had been shown to possess weak antifungal activity (Van Etten 1976).

Resorcylic acid lactones

Unlike the previous two groups of plant oestrogens, the resorcylic acid lactones are not intrinsic components of food plants but are secondary mould metabolites of fungal species, principally *Fusarium*, e.g. *F. roseum* var. *graminearum* (*Gibberella zeae*) which are common field organisms which also proliferate in poorly stored grains, oil seeds and hay (Caldwell *et al.* 1970, Eugenio *et al.* 1970, Sherwood and Peberdy 1972, Abbas *et al.* 1984). There have been a number of detailed reviews on the chemistry (Shipchandler 1975), production and biological activity (Mirocha *et al.* 1971, 1977, Mirocha and Christensen 1974, Pathre and Mirocha 1976, Hidy *et al.* 1977, Betina 1984) of these compounds and a comprehensive coverage of these and other aspects of *Fusarium* moulds is now available (Moss and Smith 1984). The economic losses associated with the feeding, especially to swine and cattle, of rations containing such mould-damaged produce have rightly meant that emphasis is primarily placed on the effects of such compounds on livestock, rather than on humans. However, since there is, at least in principle, the possibility of these compounds being carried over into humans via the consumption of animal products, and as many grain and cereal products are now formulated directly for human consumption, it is appropriate to consider the levels of such compounds likely to enter the human body and, thereby, assess the likely risk from such compounds.

The most common oestrogen of this group is zearalenone (6-(10-hydroxy-6-oxo-*trans*-1-undecenyl) β-resorcylic acid lactone, XXIII). The reduced compound, zearalanol (XXVI, figure 8), has been marketed as a growth promoter in sheep and bovines. The main metabolites of zearalenone are the epimeric β- and α-zearalenols (XXV and XXIV). Other compounds have been identified (Verdeal and Ryan 1979), but in general little is known about their biological activity. Zearalenone is usually described as a mycotoxin (F-2 toxin) but some reviewers consider this as inappropriate (Stob 1983). There have been a number of cases reported where the feeding of *Fusarium*-infected rations have caused death, abortion and other serious physiological disorders in livestock and poultry, and in some cases these were attributed to the presence of zearalenone. It is possible that other, more toxic, substances were also present, since such moulds normally produce a number of mycotoxins simultaneously; these may include trichothecenes, such as T-2 toxin, deoxynivalenol and diacetoxyscirpenol. As Stob (1983) has stated, the involvement of zearalenone in some of the more distressing symptoms associated with the feeding of *Fusarium*-infected rations should be treated with circumspection and the role of zearalenone itself should be demonstrated in controlled feeding trials, where such additional compounds can be excluded. The observed LD_{50} of zearalenone is certainly far removed from those of other mycotoxins, being 5, 10 and 20 g/kg in female guinea pigs, rats and mice respectively. For these and other reasons, Stob (1983) has suggested the terms 'mycoestrogen', 'fungal oestrogen' or 'oestrogenic metabolite' as being more appropriate.

Zearalenone, zearalenol and zearalanol have been found to bind to mammalian

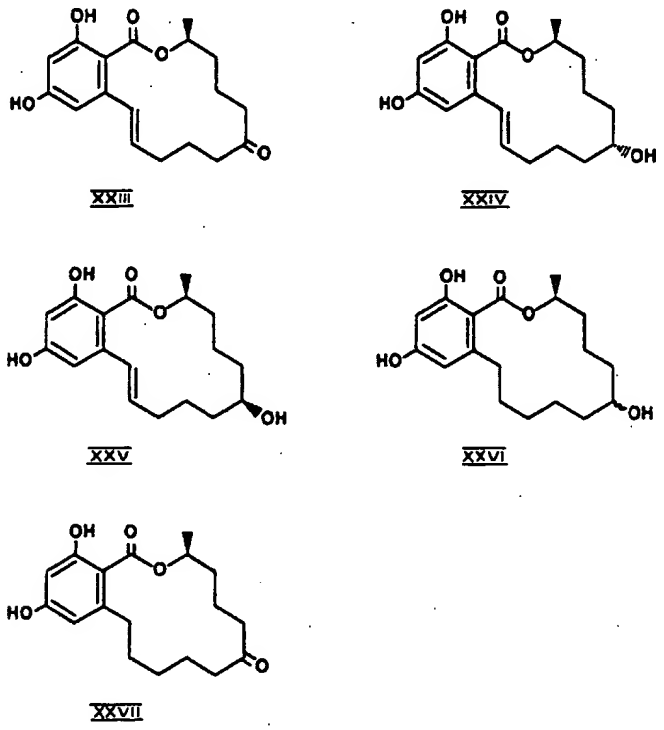


Figure 8.

oestrogen receptor sites. Kiang *et al.* (1978) showed these to bind to uterine cytosol and nuclear receptors in the order *cis*-zearalenone (not naturally occurring) > *trans*-zearalenone > zearalenol (stereochemistry unspecified) > zearalanol. All four compounds almost completely inhibited the binding of 17β-oestradiol at a ratio of 100:1. Katzenellenbogen *et al.* (1979) found α-zearalenol to be more active than either β-zearalenol or zearalanone when measured by competitive or direct binding assays using rat uterine cytosol receptors. The former compound was observed to possess 13·6% and 15% of the effect of 17β-oestradiol upon competitive and direct binding analysis, respectively.

It has been suggested (Ueno and Tashiro 1981) that the oestrogenic effect of zearalenone is due to its metabolism to zearalenol, and this suggestion has been supported by more recent work (Sheehan *et al.* 1984). Despite the structural dissimilarity between zearalenone and 17β-oestradiol, as Duax *et al.* (1984) have pointed out, there is considerable similarity between their respective hydrophobic bulk. Zearalenone binds to rat hepatic cytosol oestrogen receptors (Powell-Jones *et al.* 1981) as well as to those of rat uterus, with which it has been found to bind more strongly than the isoflavones but less strongly than coumestrol (Verdeal *et al.* 1980). Radio-labelled zearalenone, injected intravenously into mice, was found to be bound to oestrogen target organs, e.g. uterus, intestinal testicular cells and ovarian follicles (Appelgren *et al.* 1982). Studies by Martin *et al.* (1978) showed that zearalenone was less potent than

either isoflavones or coumestrol when assayed by competitive binding to human breast cancer cell oestrogen receptors. The uterotropic activity of zearalenone has been demonstrated by various workers (Stob 1983)—for example, when administered by mouth it was 10^3 times less active in the mouse than was diethylstilboestrol (figure 9). By subcutaneous injection in the same species, the compound was 500 times less active than 17β-oestradiol (Katzenellenbogen *et al.* 1979). Mirocha *et al.* (1978) have shown *cis*-zearalenone to possess stronger uterotropic activity than the natural *trans*-isomer; *cis*- and *trans*-zearalenols were found to be of comparable activity by the same workers.

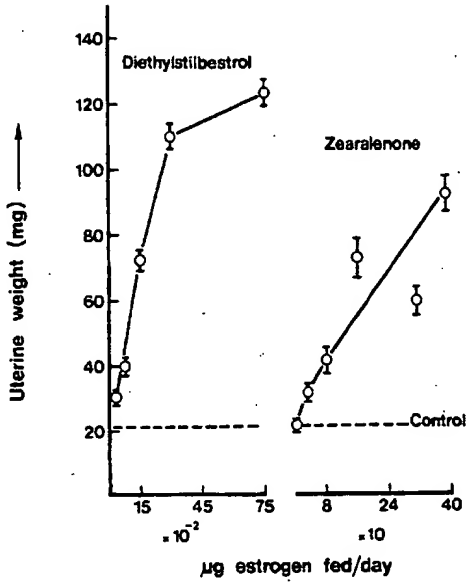


Figure 9. Relative uterotropic activity of orally administered diethylstilboestrol and zearalenone (after Stob 1983).

The biological activity of zearalenone in animals is shown in table 4. Data on the oestrogenic potency of zearalenone in rats has been obtained by Kumagai and Shimizu (1982); by uterotropic assay this compound possessed less than 0·1% of the activity of 17β-oestradiol, a figure in agreement with that resulting from estimation of vaginal cornification following systemic administration. In contrast, the vaginal cornification bioassay indicated that zearalenone possessed about 1% of the activity of 17β-oestradiol when administered locally. Furthermore, neonatal exposure to zearalenone produced anovulatory sterility in the rat, the potency being 10% that of 17β-oestradiol. There is considerable evidence that pigs are especially sensitive to zearalenone (Mirocha *et al.* 1974, Chang *et al.* 1979) with hormonal effects resulting from as little as 1–5 mg/kg diet. According to Chang *et al.* (1979), the inclusion of 25–100 mg zearalenone/kg in the ration of sows led to multiple reproductive deficiencies, including infertility, reduced litter size and weight and hyperoestrogenism. Zearalenone and zearalanol have been shown to increase weight gain when implanted subcutaneously in sheep (Hidy *et al.* 1977) and subsequent study demonstrated the latter to be especially

effective in steers and heifers (Willemart and Bouffault 1983). Further work also resulted in synthetic zearalanol implants being produced commercially.

In primates, orally administered zearalenone was 80 or 160 times less active than 17β-oestradiol or diethylstilboestrol, respectively, in inhibiting synthesis and release of gonadotropins (luteinizing hormones) from the anterior pituitary (Hobson *et al.* 1977), but the effect is much greater if the compounds are administered by subcutaneous injection and all three compounds are then of comparable effectiveness. In humans, 75–100 mg of zearalenone/day have been reported to be effective in the treatment of post-menopausal syndrome in women (Utian 1973, Hidy and Baldwin 1976b) and, according to Hidy *et al.* (1977), the clinically effective daily dose of zearalanol for such purposes is 50–75 mg, a practical result which was in agreement with that inferred from animal experiments. Both zearalanol and zearalenone are effective as oral contraceptive agents in humans (Hidy *et al.* 1976a).

According to unpublished work cited by Hidy *et al.* (1977), the major metabolite of orally administered zearalenone in the sheep was β-zearalenol (XXV) which was shown to possess only 25% of the oestrogenic activity of the parent compound. Ueno and Tashiro (1981) observed only small amounts of this metabolite in rat faeces, the main product being the epimer, α-zearalenol (XXIV), which was three times more uterotropically active than zearalenone and also bound more strongly to mammalian uterine cytosol receptor. α-Zearalenol may be present in both the free and conjugated (β-glucuronate) forms (Kiessling and Pettersson 1978, Olsen *et al.* 1981) and conjugation of zearalenone itself may also represent a significant detoxification process (Kiessling and Pettersson 1978, Olsen *et al.* 1981) in the rat. Rumen microbes have been observed to metabolize zearalenone into α- (mainly) and β-zearalenols and it has been suggested that an additional explanation of the oestrogenic effect of zearalenone-zearalenol metabolism is the interruption of normal steroid metabolism via the necessary involvement of hydroxysteroid dehydrogenase (Kiessling and Pettersson 1978, Olsen *et al.* 1981). As has been mentioned earlier, zearalanol has been used to improve weight gain of livestock. The main metabolite of this compound in a wide range of species, including man, is zearalanone (XXVII); both compounds have been found in the free and bound forms (Hidy *et al.* 1977).

According to Dixon and Russell (1983), when four cattle were implanted with 36 mg of zearalanol, mean maximum urine levels were 13·5 μg/l (22 days after implantation) and declined to 2·9 μg/l by day 69. Two experiments were conducted with sheep, who received 12 mg zeralanol implanted into the base of the ear. Mean maximum urine levels were reached after 35 days (45 μg/l) and 56 days (90 μg/l) in the two experiments, and thereafter declined to 26 μg/l (day 42) and 11·7 μg/l (day 70), respectively. Ruddick *et al.* (1976) reported zearalenone to be teratogenic although more recent work (Davis *et al.* 1977, Wardell *et al.* 1982) could not confirm this.

Numerous methods have been described for the analysis of zearalenone (Gilbert 1984), including thin layer and paper chromatography (with colourimetric or ultraviolet detection) (Caldwell *et al.* 1970), gas chromatography (utilizing the trimethylsilyl- or pentafluoropropionate derivatives) (Steele *et al.* 1976, Holder *et al.* 1977), gas chromatography-mass spectrometry (Mirocha *et al.* 1974, Scott *et al.* 1978) and high-performance liquid chromatography (Scott *et al.* 1978, Cohen and Lapointe 1980). Thin layer and high-performance liquid chromatography have also been used to separate zearalenone and its metabolites in biological samples (Kiessling *et al.* 1984, Ueno and Tashiro 1981). The detection limit for zearalenone using high-performance liquid chromatography, with Spherisorb 5 μm column and fluorescence detection, was

5 μg/kg in corn flakes (although a second high-performance liquid chromatography column was needed to remove an interfering compound in other corn products) and 10 μg/kg in corn (Scott *et al.* 1978, Ware and Thorpe 1978, respectively). For purposes of routine screening, simpler techniques using thin layer chromatography have been developed. With Fast Violet B as spray reagent, detection limits of 20 μg/kg (Scott *et al.* 1978) and 80 μg/kg (Swanson *et al.* 1984) have been reported for zearalenone in corn and corn-based foods. The latter workers also considered the method amenable for the qualitative, but not quantitative, screening of zearalenol (detection limit 200 μg/kg). Immunological techniques have been developed for the detection and quantification of zearalanol (Dixon 1980, Dixon and Russell 1983, Thouvenot and Morfin 1983) but since related compounds, such as zearalanone and zearalenone, may possess significant cross-reactivity towards the antiserum, a preliminary separation with high-performance liquid chromatography has been recommended (Jansen *et al.* 1984).

The extent of the contamination of grain crops with *Fusarium* species may be considerable. In 1972, 38 out of 223 corn samples from areas in the USA where such contamination was suspected or expected were found to contain zearalenone, the levels ranging from 100 to 5000 μg/kg (Eppley *et al.* 1974). A similar study the following year revealed zearalenone levels of 38–294 μg/kg in 19 out of a total of 315 marketable corn samples (Stoloff *et al.* 1976). There was clear evidence of localized regional occurrence with 10% of the samples from the Corn Belt (17 out of 169) being affected. The same workers also measured zearalenone contents of 97–10 400 μg/kg in 57 samples of obviously damaged corn. The results of other surveys of wheat, grain sorghum, soyabeans and corn have been summarized by Bennett and Shotwell (1979). Zearalenone has been detected in six samples of Mexican corn intended for human consumption, but the levels were not quoted (Mirocha *et al.* 1972). Of 293 samples of the 1982 Australian maize crop recently examined (Blaney *et al.* 1984), 85% contained zearalenol; the mean concentration was 170 μg/kg but four samples possessed in excess of 1000 μg/kg. Côté *et al.* (1984) found 40 out of 342 feed samples, obtained in 1981 from the area around Illinois and suspected of causing or contributing to animal health problems, to contain zearalenone. Levels ranged from 100 to 8000 μg/kg, with a mean of 660 μg/kg. In Canada, problems associated with *Fusarium* infection of corn and other crops would seem to occur predominantly in Ontario (Andrews *et al.* 1981). Analysis of suspected samples over the period 1972–1977 revealed some 10% (214 out of 2022) to possess zearalenone, levels ranging from 10 to 141 000 μg/kg, the mean being 3850 μg/kg. Zearalenone, deoxynivalenol and, apparently for the first time, aflatoxin B_1, have recently been identified in commercial wheat samples from the mid-western USA. Of a total of 33 samples examined, zearalenone was present in trace amounts in two samples and, in another three, at levels of 35, 90 and 115 μg/kg (Hagler *et al.* 1984).

There is some disagreement over the effectiveness of chemical treatments for detoxification of zearalenone-contaminated grain. An American patent (Tamas and Wöller 1977) describes either 3–6% aqueous hydrogen peroxide or ammonium hydroxide as effective, but the removal of zearalenone was not quantified. However, unpublished work, referred to by Bennett and Shotwell (1979), found the ammoniation process used for removal of aflatoxins to have no effect on zearalenone levels. More recently, Kallela and Saastamoinen (1981) have shown the farm grain preservative 'Gasol' to have a beneficial effect in reducing the levels of zearalenone in stored grains.

A considerable amount of the world grain crops is used as human food sources, either directly or after processing. In many parts of the world such use represents the

major part of the crops' utilization. There have been a number of reports of zearalenone being found in southern African foods, drinks and raw materials. Thus levels of 100–800 μg/kg were measured in corn used for the brewing of Zambian beer (Lovelace and Nyathi 1977) with an average of 920 μg/kg (maximum 4600 μg/kg) being found in such beers and 800–4000 μg/kg in the corn malt used in the brewing process. Of 55 samples of sour drinks, porridges and beers from Swaziland, six were found to contain zearalenone (referred to in Bennett and Shotwell 1979) at levels between 800 and 5300 μg/kg. Of local beers from Lesotho, 12% of the 140 samples examined also contained this oestrogen (300–2000 μg/kg). Rather lower levels were found in Lesotho beer by Martin and Gilman (1976) (approximately 50 μg/kg) and samples of maize porridge, sorghum malt were also found to be contaminated (Martin 1974). MacDonald and Raemakers (1974) found zearalenone in South African maize samples. Together with zearalenone, the presence of other, more toxic, metabolites may be expected (Bennett and Shotwell 1979) and although the climate in southern Africa might be expected not to be such as to facilitate such mould growth as might occur in other parts of the world, Marasas *et al.* (1977) have found strains of *F. graminearum* in southern Africa capable of producing deoxynivalenol, and possibly other mycotoxins.

According to Stoloff and Dalrymple (1977), zearalenone was not detected in the primary or by-products from dry milling operations. Bennett *et al.* (1976, 1978) have examined the effects of processing on naturally contaminated corn. Wet milling was found to concentrate the oestrogen in the gluten fraction with lesser amounts being found in the milling solubles, fibre and germ respectively. The starch fraction was free of zearalenone. Dry milling led to a two- to three-fold concentration of the zearalenone in the germ. Both milling processes led to a concentration of the zearalenone into fractions used as animal feedingstuffs.

Scott *et al.* (1978) have examined various corn products for zearalenone using both high-performance liquid chromatography and gas chromatography. Largest amounts were found in a sample of cornmeal (26 μg/kg), although two other samples contained no detectable amounts. Frozen corn contained 2 μg/kg, corn chips 0 and 2 μg/kg, popcorn 0 and 7 μg/kg and three samples of cornflakes 0, 0·4 and 14 μg/kg respectively. The carry-over of zearalenone in cattle consuming naturally infected wheat rations has been studied by Shreeve *et al.* (1979). Concentrates (385–1925 μg zearalenone/kg) were fed to two cows for 7 weeks. No zearalenone (detection limit 4 μg/kg) residues were detected in muscle, kidney, liver, serum, milk or urine. The result should be interpreted with some caution bearing in mind the number of animals used and the inability of the analytical method used to detect zearalenone metabolites. The study also revealed apparent indications of interactions between dietary fungal metabolites which would warrant further examination. Mirocha (1981) has detected α- and β-zearalenol (16–76 μg/kg) in the milk of a cow following the oral dosage of [^{3}H]zearalenone. Palyusik *et al.* (1980) have described the results of feeding two lactating sows a diet containing pure zearalenone (40 mg/kg). In addition to various physiological effects attributable to the oestrogenic effect of this compound, analysis of the milk from these animals showed mainly β-zearalenol (> 80% of original toxin) and α-zearalenol (~15%) with only traces (0·5–1·3%) of unchanged zearalenone. The highest concentration of zearalenol found in milk was 0·79 p.p.m. The authors reported that the metabolites could be detected in the milk samples within 2 days of feeding the zearalenone and were still present 5 days after it had been removed from the diet.

Calculations quoted by Lovelace and Nyathi (1977) give possible daily intakes of zearalenone of 450 μg and 170 μg for rural farmers in Southern Province and

inhabitants of Lusaka, respectively. The figure for certain individuals is certainly much higher. Marasas *et al.* (1979), on the basis of animal data, considered that 500 μg/kg was a biologically significant dose of zearalenone. However, as has been indicated, there is a considerable variation in sensitivity between species and the toxicity of zearalenone in man is unknown, but based upon data from other primates (Hobson *et al.* 1977) is probably low. Ueno and Kubota (1976) suggested that zearalenone was mutagenic to a recombination-deficient line of *Bacillus subtilis*, but this could not be confirmed by Wehner *et al.* (1978), using *Salmonella typhimurium*.

Schoental (1979) has suggested that zearalenone and other *Fusarium* mycotoxins may have a role in the aetiology of tumours of the digestive tract and gonads in animals and man, and there has been speculation (see Martin and Keen 1978) that dietary oestrogens might be implicated in the high incidence of cervical cancer in certain areas of Southern Africa, e.g. Swaziland and Lesotho.

Mouldy corn from areas of the Transkei associated with high and low incidences of oesophageal cancer has been examined for mycotoxins (Marasas *et al.* 1979). Pooled samples from these areas showed no significant differences in the extent and nature of the *Fusarium* species present. However, when four sub-samples of hand-selected, visibly *Fusarium*-infected kernels were analysed, significant differences in the nature and extent of the infection were observed. All of the sub-samples contained zearalenone (ranging from 1500 to 10 000 μg/kg) but the mean level of the samples from the high-incidence area was 5750 μg/kg compared to 2750 μg/kg from the low-incidence area. Even larger differences were noted in the levels of deoxynivalenol, being 250 μg/kg and 2500 μg/kg in the low-incidence and high-incidence areas respectively. The authors concluded that before the potential threat to human health of these *Fusarium* metabolites in mouldy corn could be evaluated more detailed information was needed on their chronic effects and on whether any additive or synergistic effects might occur.

Other compounds claimed to possess oestrogenic activity

Examination of table 1 reveals additional compounds which have been claimed, with varying degrees of supportive evidence, to be responsible for the oestrogenic activity of the individual plant species shown. For example, Stob (1983) has suggested that the hormonal activity of carrots (Ferrando *et al.* 1961) may be related to the presence of 3-methyl-6-methoxy-8-hydroxy-3,4-dihydroisocoumarin (XXVIII, figure 10). However, this compound was isolated from cold-stored carrots and was apparently absent in the freshly harvested root (Sondheimer 1957). Little, if anything, is known about the effect of such storage on the uterotropic effect of this vegetable. Anethole (XXIX) was suggested by Zondek and Bergmann (1938) to be responsible for the oestrogenic activity of essential oils of fennel and anise, but more recent physiological studies on this compound (Sangster *et al.* 1984a, b) have not supported this. Three structurally related bitter acids, colupulon (XXX), lupulon (XXXI) and adlupulon (XXXII) have been identified in hops and proposed as the oestrogenic principles therein (Zenisek and Bednar 1960).

Feldman *et al.* (1982) described a protein in bakers' yeast (*Saccharomyces cerevisiae*) capable of binding 17β-oestradiol with high affinity; moreover, a chloroform extract of the same yeast cells was found to bind competitively to mammalian oestrogen receptor cells *in vitro*. Subsequently the same group (Feldman *et al.* 1984) showed this extract to possess uterotropic activity. If these findings are confirmed, high priority should be given to the isolation and identification of the active component(s), given the extensive use of this material in baking and fermentation. Only when its potency has

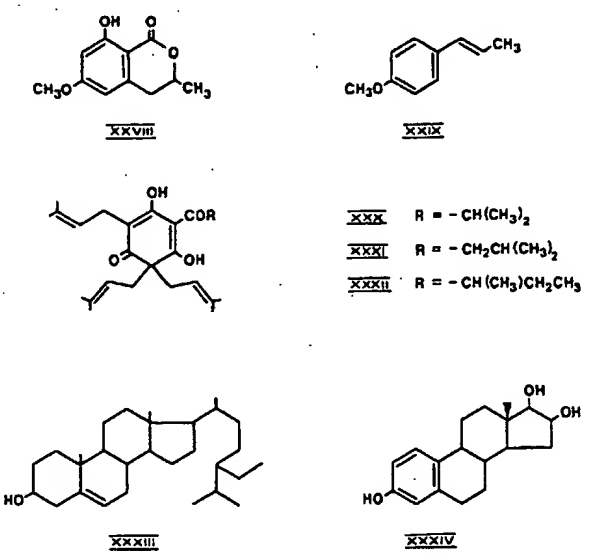


Figure 10.

been determined and its levels in food products have been established can the full significance of the above findings be ascertained.

According to Hassan *et al.* (1964), β-sitosterol (XXXIII) was one of the factors responsible for the hormonal activity of liquorice, although this has been questioned. Certainly if this and related compounds were to be confirmed as possessing such activity, then their ubiquity might well explain the hormonal properties of a range of common food plants, including onion and garlic and certain vegetable oils (Booth *et al.* 1960). Rather earlier, Costello and Lynn (1950) had tentatively identified the steroidal oestrogen, oestriol (XXXIV), as being present in liquorice. The role, and indeed the presence, of such steroidal hormones in the plant kingdom has been the subject of considerable controversy (Hewitt *et al.* 1980). As these workers have emphasized, early investigations into the hormonally active principles of plants were limited by relatively crude means of fractionation, isolation, characterization and bioassay. Consequently many of the initial claims for the occurrence of steroidal oestrogens in the plant kingdom were treated with scepticism. In 1966, Bennett *et al.* identified oestrone in date palm pollen by thin layer chromatography, and it was subsequently isolated from the same source (Amin *et al.* 1969). Pomegranate seed was claimed to be another source of this compound, with levels of 17 mg/kg being reported (Heftmann *et al.* 1966).

Whilst the presence of oestrone was confirmed by Dean *et al.* (1971), the measured levels were very much lower (4 μg/kg). 17β-Oestradiol could not be detected. In other cases (see Hewitt *et al.* 1980), workers were unable to isolate steroidal oestrogens from plant sources despite previous claims to the contrary. Such irreproducibility, limitations in analytical technique and methodology and the overriding concern that presence of such compounds, even when proved conclusively, might be a result of contamination meant that fundamental questions about the natural occurrence of such compounds in plants remained until recently (Van Rompuy and Zeevaar 1979), and the

failure of these workers to identify steroidal oestrogens in plant extracts using sophisticated modern techniques clearly identifies this area as a rewarding one for further interdisciplinary study. Work described in detail by Hewitt *et al.* (1980), using radioisotope incorporation studies and sensitive gas chromatography-mass spectrometric techniques, unequivocally revealed the presence of oestrone and oestradiol in French bean seedlings.

Residues of pesticides and insecticides may also be a source of uterotropically active compounds in the human diet. It has long been known that DDT and its analogues exhibit such activity (Fisher *et al.* 1952, Welch and Conney 1968) and recently Loeber and van Velsen (1984) have shown β-HCH, an isomer of lindane and a component of technical HCH, to have uterotropic activity. Although very weak (2×10^{-5} that of 17α-ethynyloestradiol), little is known about the effects of long-term exposure to trace amounts of such compounds.

Overview

Amongst the plants consumed by humans which have been reported to possess oestrogenic activity are onion, garlic, coffee, apple, parsley, sage, rhubarb, potato, radish, pea, cucumber, sugar beet, cabbage and mustard. These reports, originating from the early work of Dohrn *et al.* (1926) and Löve and Löve (1945), did not identify any of the active components, and were based upon methods of analysis which are now recognized to have limitations. Nevertheless it would seem desirable to re-examine some of these food plants using modern methods of analysis, in particular those like potato and cabbage, which are consumed regularly in relatively large amounts. The widespread use of vegetable oils also suggests that the claims that these are uterotropic be re-examined. Because of the evident variation in sensitivity to oestrogens exhibited by different species and strains of animal it would be desirable to standardize the uterotropic assay so that results from different laboratories and on different commodities/food plants could be more readily compared.

Inspection of table 6, taken from Verdeal and Ryan (1979), would seem to suggest that there is little risk associated with the intake of plant oestrogens. This is not necessarily the case, however, since little is known about the effects of long-term low-level exposure to these compounds (or their metabolites). Studies with human subjects would be desirable to determine whether or not normal levels of intake are associated with detectable physiological changes. This might provide objective predictions of the nature and extent of any changes which might occur in particular

Table 6. Human exposure to exogenous oestrogens.[a]

Source	Estimate of possible daily dose (μg diethylstilboestrol equivalents)
Morning-after pill	50 000
Birth control pill	2500
Post-hysterectomy replacement therapy	500–1000
Post-menopausal therapy	500
100 g beef liver (0·5 p.p.b. diethylstilboestrol)	0·05
100 g wheat (2 p.p.m. zearalenone)	0·2
20 g (d.w.) soyabean sprouts (70 p.p.m. coumestrol)	0·5
100 g French beans (2–10 p.p.b. oestradiol)	0·03–0·15

[a] Data from Verdeal and Ryan (1979) with permission.

'at-risk' sections of the population. Moreover, consideration should be taken of any medium or long-term changes in dietary habits which might be expected to increase the intake of such phytoestrogens; the increasing use of vegetable proteins in general and soya protein in particular and the introduction of soya milk products for infant feeding are two such examples (Setchell *et al.* 1984).

The importance of metabolic studies in determining the likely oestrogenic effect associated with the ingestion of isoflavones and resorcylic acid lactones is obvious, the effect depending as it does on the extent of that metabolism and the individual potencies of the metabolic products. The metabolism of the coumestans should be examined and the activities of the major isolated metabolites determined. Further studies should also be conducted on equol; for example, examining its effect *in vivo* and *in vitro*. Such studies will obviously depend upon the availability of methods of analysis for both the parent compounds and the metabolites. The examination of the possible carry-over of resorcylic acid lactones, and metabolites, following the feeding of *Fusarium*-infected diets or the implantation of zearalanol as growth stimulant would be desirable using such methods. The possible synergistic effect of different plant oestrogens or of plant and synthetic oestrogens should not be discounted (Kotsonis *et al.* 1975).

Attention should be given to programmes designed to limit or reduce the intake of plant oestrogens, whether by judicious selection of plant varieties or the optimization and improvement of agronomic, storage and processing conditions. Notwithstanding the inherent difficulties, a detailed study of the dietary factors associated with the high incidence of certain cancers in Southern Africa might provide useful information as to the role of dietary oestrogens. Indeed, a fuller understanding of the biological basis of the hormonal activity of the plant oestrogens considered above, particularly in farm animals and primates, would seem long overdue.

Future progress in this interesting and challenging area will largely depend upon the integrated efforts of workers from a variety of disciplines, including chemists, biochemists, toxicologists, pathologists, food technologists and plant breeders. As such it would seem to be particularly suitable for support from national and international food and health agencies.

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